

REGULATION OF THE GERMINAL CENTER REACTION BY T
HELPER CELLS AND T REGULATORY CELLS

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REGULATION OF THE GERMINAL CENTER REACTION BY T HELPER CELLS AND T REGULATORY CELLS

Germinal Centers (GCs) are transient lymphoid structures that arise in lymphoid organs in response to T cell-dependent antigen. Within the GC, follicular T helper (TFH) cells promote GC B cell differentiation and in turn the proper antibody production to protect us from invading pathogens. We wished to study the regulation of this process by transcription factors STAT3 and Bcl6. STAT3 is important for both TFH cell differentiation and IL-4 production by Th2 cells. IL-4 is a major functional cytokine produced by TFH cells. To dissect the role of STAT3 in IL-4 production by TFH cells, we generated T cell-specific conditional STAT3 knockout mice (STAT3KO). Compared to WT mice, TFH cell differentiation in STAT3KO mice was partially impaired, both in spleen following sheep red blood cells (SRBC) immunization and in Peyer's patches (PPs). In STAT3KO mice, the numbers of splenic GC B cells were markedly decreased, whereas PP GC B cells developed at normal numbers and IgG1 class switching was greatly increased. Unexpectedly, we found that STAT3 intrinsically suppressed the expression of IL-4 and Bcl6 in TFH cells. Mechanistically, in vitro repression of IL-4 expression in CD4 T cells by Bcl6 required STAT3 function. Apart from TFH cells, the GC reaction is also controlled by regulatory follicular T helper (TFR) cells, a subset of Treg cells. To study the mechanism of how TFR cells regulate the GC reaction, we generated mice specifically lacking TFR cells

by specifically deleting Bcl6 in Treg cells. Following immunization, these “Bcl6FC” mice developed normal TFH and GC B cell populations. However, Bcl6FC mice produced altered antigen-specific antibody responses, with reduced titers of IgG and increased IgA. Bcl6FC mice also developed IgG antibodies with significantly decreased avidity to antigen in an HIV-1 gp120 “prime-boost” vaccine model. Additionally, TFH cells from Bcl6FC mice produced higher levels of Interferon- γ , IL-10 and IL-21. Loss of TFR cells therefore leads to highly abnormal TFH and GC B cell responses. Overall, our studies have uncovered unexpected regulatory roles of STAT3 in TFH cell function as well as the novel regulatory roles of TFR cells on cytokine production by TFH cells and on antibody production.

Alexander L. Dent, Ph.D., Chair

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List of Abbreviations

AID	Activation-induced deaminase
APC	Antigen presenting cell
Bcl6	B cell lymphoma 6
Bcl6FC	Bcl6 ^{fl/fl} Foxp3 ^{cre} mice
BCR	B cell receptor
Blimp1	B lymphocyte-induced maturation protein-1
BoyJ	B6.SJL-Prprc ^a Pepc ^b /BoyJ
CD40L	CD40 ligand
CXCL13	C-X-C motif chemokine 13
CXCR5	C-X-C motif receptor 5
DC	Dendritic cell
dpi	Day post immunization
Fas	TNF receptor superfamily member 6
FDC	Follicular dendritic cell
GATA3	GATA binding protein 3
GC	Germinal center
HIES	Hyper-IgE syndrome
ICOS	Inducible T-cell costimulator
ICS	Intracellular staining
IFN-γ	Interferon-gamma

Ig	Immunoglobulin
IL	Interleukin
IRF4	Interferon regulatory factor 4
JAK	Janus kinase
MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
OVA	Ovalbumin
PD-1	Programmed cell death protein 1
pMHC I	MHC I-peptide complex
pMHC II	MHC II-peptide complex
PP	Peyer's Patch
QPCR	Quantitative RT-PCR
RORα	Retinoic acid receptor-related orphan receptors alpha
RORγ	Retinoic acid receptor-related orphan receptors gamma
RV	Retrovirus
SAP	SLAM-associated protein
SLAM	Signaling lymphocyte activation molecule
SLE	Systemic lupus erythematosus
SRBC	Sheep red blood cell
STAT	Signal transducer and activator of

	transcription
STAT3FC	STAT3 ^{fl/fl} Foxp3 ^{cre} mice
STAT3KO	CD4-cre STAT3 ^{fl/fl} mice
TCR	T cell receptor
TFH	Follicular T helper cell
TFR	Regulatory follicular T helper cell
TGF-β	Transforming growth factor beta
Th	T helper cell
Treg	T regulatory cell
TSLP	Thymic stromal lymphopoietin

Chapter 1 - Introduction

1.1: Innate immune system.

In vertebrates, including human and mouse, the immune system can be divided into two cooperative components: innate immune system and adaptive immune system. The immune system protects us from bacterial, viral and fungal infections. The innate immune system is an evolutionarily ancient and universal host defense mechanism [1]. It is present in all multicellular organisms including plants and insects. The activation of the innate immune system relies on the recognition of pathogen-associated molecular patterns by pattern recognition receptors expressed on the surface or in the cytoplasm of innate immune cells [2]. The innate immune system has two main functions [3]. First, it is the front line of host defense providing the rapid initial response to control and eliminate infections. Second, the innate immune system can activate and modulate adaptive immunity through antigen presentation, cell-cell contact and secretion of cytokines.

The innate immune system is made of multiple components. The first and outmost line of innate defense is the anatomical barriers. Skin and mucosal surfaces in gastrointestinal and respiratory tracts are crucial for protecting us from the infection of pathogenic microbes. The second line of innate defense is the innate immune cells, mostly derived from the common myeloid progenitor cells [4], including dendritic cells (DCs), macrophages, monocytes, mast cells,

neutrophils, eosinophils and basophils. Working together, these cells help protect us from the majority of the infections.

Depending on the immune environment, monocytes can further differentiate into DCs or macrophages [5, 6] which are professional antigen presenting cells (APCs). They can engulf the invading bacteria or virus infected cells by phagocytosis, then present the antigen to the T cells to initiate the adaptive immune response. They function as messengers between innate and adaptive immunity.

Neutrophils, eosinophils, mast cells and basophils are granulocytes, each of them having distinct granules in their cytoplasm. Neutrophils, the most abundant type of granulocyte, quickly migrate towards the infection site by sensing the chemical gradients of molecules such as interleukin-8 (IL-8) and Leukotriene. Neutrophils can phagocytose microbes or particles that are opsonized by antibodies. In addition, neutrophils secrete cytokines and antimicrobial contents by degranulation, which in turn recruit other immune cell types to amplify the inflammatory response [7]. Granules in eosinophils contain many chemical mediators, such as peroxidase, ribonuclease, leukotrienes, prostaglandin, growth factors and inflammatory cytokines which make eosinophils capable of combating against parasitic and viral infections [8]. Even though mast cells reside in the tissue whereas basophils are in the circulation, these two cell types are very similar in morphology and function. Both cell types have granules containing histamine and heparin which are ready to be released upon activation. Degranulation of mast cells and basophils can be activated by

the binding of surface receptors to the immunoglobulin E (IgE)-allergen complexes. Therefore, mast cells and basophils play critical roles in allergy, asthma and many other allergic diseases [9, 10].

1.2: Adaptive immune system and T helper cell subsets.

Since surface receptors like pattern recognition receptors and other protein molecules involved in the innate immunity are encoded by the germline, the innate immune system is fixed, rigid and not adaptable [3]. Pathogens develop ways to evade it. Lymphocytes, T and B cells, which comprise the adaptive immune response, overcome this disadvantage with the specificity of T cell receptors (TCR) and B cell receptors (BCR). During the early stage of lymphocyte development, genetic recombination of DNA encoded gene segments generate TCRs and BCRs in a quasi-random manner mediated by the recombination activating genes 1 and 2 (Rag 1 and Rag2) [11]. At the same time, T cells in the thymus and B cells in the bone marrow undergo positive and negative selections so that they can recognize foreign but not self antigens. This mechanism creates an extremely diverse repertoire of T and B cells that are able to recognize millions of different foreign antigens.

The two main subpopulations of T cells are CD8⁺ and CD4⁺ T cells. CD8⁺ T cells, or cytotoxic T cells are specialized to kill cancer cells and cells infected with intracellular pathogens. Naive CD8⁺ T cells require the TCR recognition of the specific antigen peptide presented in the major histocompatibility complex I (MHC I) molecules by APCs and the co-stimulatory signaling to get activated. All

nucleated cells express MHC I, giving the cells ability to present pathogenic antigen peptide to CD8⁺ T cells. During infections, once the TCRs on the activated cytotoxic CD8⁺ T cells recognize the MHC I-peptide complex (pMHC I) on the infected cells, the targeted cells will be destroyed.

CD4⁺ T cells, or T helper cells, provide help to other immune cells such as CD8⁺ T cells, B cells and APCs. Similar to the CD8⁺ T cell activation, naive CD4⁺ T cells get activated by APCs through the TCR recognition of MHC II-peptide complex (pMHC II). Depending on the type of infection, APCs can produce varieties of cytokines and in turn induce CD4⁺ T cell differentiation into several subsets, type 1 T helper (Th1), Th2, Th9, Th17, follicular T helper (TFH) and regulatory T helper (Treg) cells (Fig. 1) [12]. Each subset has its own differentiation program driven by its specific master transcription factor.

Th1 cells are generated during infection with intracellular pathogens such as viruses and bacteria. IL-12 promotes Th1 cell differentiation by activating signal transducer and activator of transcription 4 (STAT4) signaling [12]. STAT4 further induces the expression of the Th1-master transcription factor, Tbet. STAT4 and Tbet work together to promote the differentiation of Th1 cells and to drive the expression of Th1 genes, such as interferon gamma (IFN- γ), which is a key cytokine for virus clearance. However, uncontrolled Th1 cell response can result in chronic inflammation.

Th2 cells mediate the immune response to large extracellular pathogens. Th2 cell differentiation requires the presence of IL-4 and IL-2 or thymic stromal lymphopoietin (TSLP) [13]. IL-4 induces STAT6-dependent expression of GATA

binding protein 3 (GATA3), which is the master transcription factor of Th2 cells. GATA3 activates IL-5 and IL-13 expression, while suppressing the differentiation of other T helper cell subsets [14]. In addition, STAT5 signaling downstream of IL-2 or TSLP can cooperate with GATA3 to promote Th2 cell differentiation by enhancing IL-4 production [14].

Th9 cells secrete high levels of IL-9 and IL-10. In the presence of transforming growth factor beta (TGF- β) and IL-4, PU.1 and interferon regulatory factor 4 (IRF4) are activated respectively which subsequently promote the production of IL-9 [15]. Th2 and Th9 cells are closely related, however, Th9 cells have limited ability to produce IL-4, IL-5 or IL-13. Th9 cells are also important for host defense against parasitic infections [16]. On the other hand, both Th2 and Th9 cells contribute to the onset and progression of allergic diseases, such as asthma [17].

At mucosal surfaces, Th17 cells play an important role in pathogen clearance to protect the mucosal barriers. TGF- β , IL-6 and IL-21 drive the differentiation of Th17 cells [18]. IL-6 and IL-21 activate STAT3 signaling which further up-regulates the expression of retinoic acid receptor-related orphan receptors gamma (ROR γ) and alpha (ROR α), the master transcription factors of Th17 cells. The main effector cytokines of Th17 cells are IL-17A, IL-17F, IL-21 and IL-22. Th17 cells are associated with autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and psoriasis [19].

TFH cells are specialized to provide “help” to B cells to mount effective antibody response against pathogens. TFH cell differentiation is a multistep

process and depends on the expression of the master transcription factor B cell lymphoma 6 (Bcl6) [20]. At early stage of the TFH cell differentiation, in the T cell zone of the secondary lymphoid organs, a small population of activated CD4⁺ T cells with the highest specific binding of pMHCII on APCs expresses Bcl6 and, the chemokine receptor, C-X-C motif receptor 5 (CXCR5). Depending on the cytokines present during the T cell response, STAT1, STAT3 or STAT4 can activate Bcl6 expression. STAT3, downstream of IL-6 and IL-21 signaling, is the most important one [21]. The expression of CXCR5 is important for the migration of pre-TFH cells towards the B cell follicle where C-X-C motif chemokine 13 (CXCL13), the ligand of CXCR5, is highly expressed. In addition, inducible T-cell costimulator (ICOS) signaling is critical for up-regulation and maintenance of Bcl6 expression [22]. Besides Bcl6, the transcription regulation network of TFH cell differentiation includes other transcription factors, such as achaete-scute homologue 2 (Ascl2) [23], Interferon Responsive Factor 4 (IRF4) [24], basic leucine zipper ATF-like Transcription Factor (Batf) [25] and v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog (cMaf) [26]. Once in the B cell follicle, TFH cells are essential to promote germinal center (GC) formation. CD40L on TFH cells stimulates CD40 signaling and cytokine signaling, such as IL-21 and IL-4, to GC B cells for their expansion and further differentiation into high-affinity antigen-specific antibody producing plasma cells. At the same time, GC B cells displaying the pMHCII on the surface maintain the fate of TFH cells by providing pivotal TCR and co-stimulatory signaling. However, an abnormal TFH cell response can give rise to the production of autoreactive antibody which

is the hallmark of several autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus (SLE), etc [27]. More details of the GC reaction will be discussed in the next section.

Treg cells are important for the maintenance of immune cell homeostasis including tolerance to self-antigens and suppression of exacerbated inflammatory immune responses. Treg cells can be broadly classified into natural and induced Treg cells[28]. Natural Treg cells develop in the thymus and emigrate into the periphery. Induced Treg cells develop in the periphery at gut and airway mucosa in response to food and environmental antigens, during chronic inflammation immune responses, as well as in autoimmune diseases. Regardless of the origin, Forkhead box P3 (Foxp3) is the master transcription factor for the differentiation of Treg cells. There are several molecular mechanisms by which Treg cells exert their regulatory function, cell contact mediated regulation via co-stimulatory molecules, cytolytic activity as well as secretion of immunosuppressive cytokines such as IL-10 and TGF- β [29].

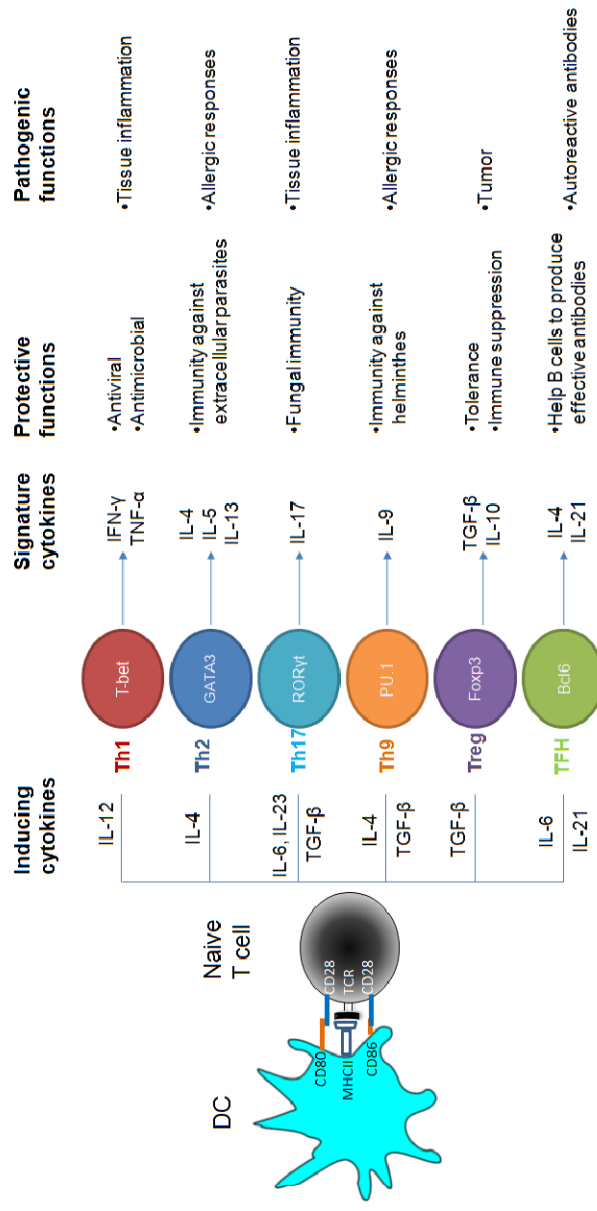


Figure 1.T helper cell subsets.

During an immune response, naive CD4⁺ T cells receive the activation signaling from DCs via TCR signaling, co-stimulatory signaling (CD80/CD86 and CD28 interactions), and cytokine signaling. Specific cytokine signaling activates the downstream master transcription factors to initiate and maintain the differentiation of corresponding T helper cell subset. Each subset secretes its own signature cytokines and exerts certain immune function to protect us from invading pathogens. However, uncontrolled T helper cell responses can lead to immunological pathologies, such as inflammation and autoimmunity.

1.3: GC.

1.3.1: GC reaction.

GCs are temporary lymphoid structures within the spleen and lymph nodes that form during T cell-dependent immune responses. The main function of GCs is to produce long lived plasma cells that can secrete high-affinity antigen-specific antibodies [30]. The general cellular process of GC reaction is summarized in Fig. 2. The timing of the GC reactions depends on the many factors, such as the types or doses of pathogen, antigen and adjuvant. Briefly, on day 0 of GC reaction, T cells and B cells recognize their cognate antigen in the T cell zone and B cell follicle, respectively. On day 1-2, activated T and B cells migrate to the T-B border area and start to interact. CD4⁺ T cells with high TCR signaling strength preferentially get into the TFH cell differentiation program [31]. T cells start to express CXCR5 which guides them into B cell follicle. In the B cell follicle, there is a network of follicular dendritic cells (FDCs) which capture immune complex as that are recognized by the antigen-specific B cells [32]. After the activation of B cells, some antigen-specific B cells differentiate into early plasmablasts, whereas other antigen-specific B cells migrate from the T-B border back into the B cell follicle and initiate the early GC on day 4 [33]. With help from TFH cells, the GC rapidly grows between day 5 and 6. On day 7 of the GC reaction, the mature GC is established, as the dark zones and light zones are formed. The light zone contains FDCs, TFH cells and GC B cells, and is the place where the B cells with the high-affinity BCRs get selected. The dark zone mainly contains densely packed GC B cells, and is the place where the BCRs

undergo extensive somatic hypermutation for further antibody maturation and class switching [30].

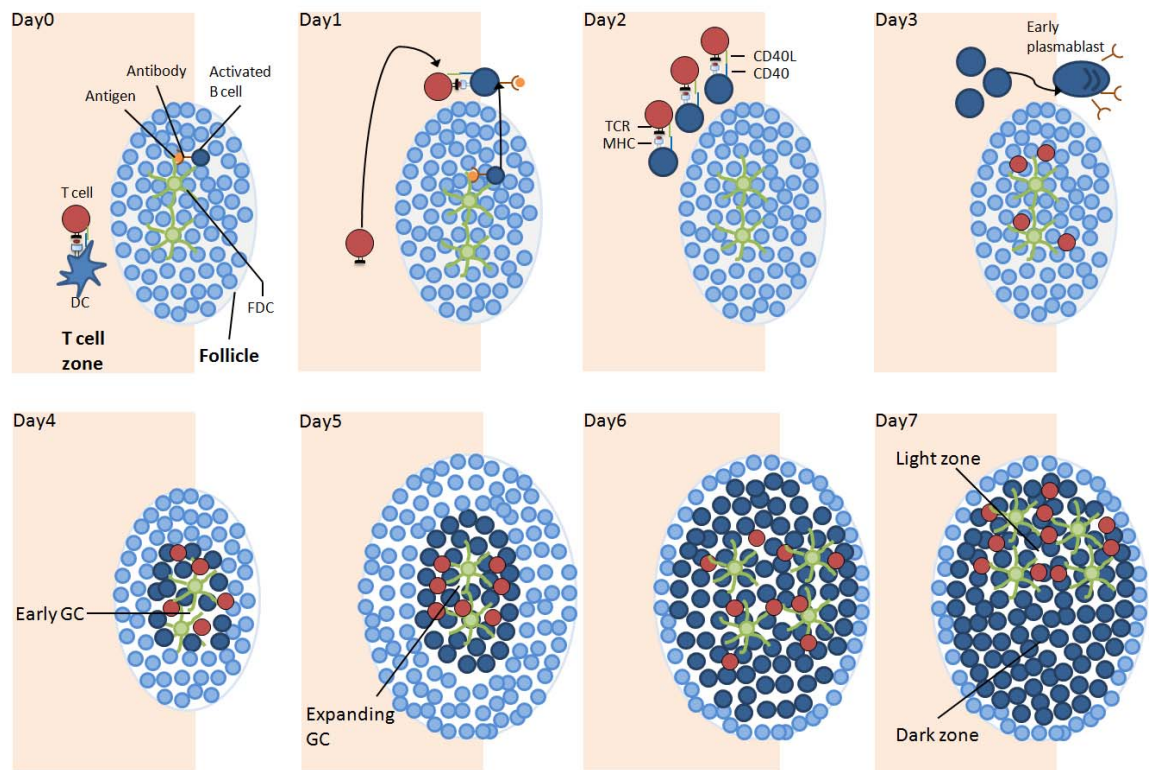


Figure 2. The formation of GC in the lymph node.

GCs develop dynamically after the activation of T and B cells. CD40L, CD40 ligand; DC, dendritic cell; FDC, follicular dendritic cell.

1.3.2: T-B cell interaction in the GC.

Cognate contact (T cells and B cells recognize the same antigen) between antigen-specific T cells and pMHCII-expressing B cells is important for both the TFH cell differentiation and the development of effective B cell immunity. The T-B cell interaction involves several cell surface molecules (Fig. 3). The members of the Signaling Lymphocyte Activation Molecule (SLAM) family of receptors, particularly CD84, play an important role in the formation of prolonged T-B cell interactions. Both in human and mice, the SLAM mediated SLAM-Associated Protein (SAP) signaling is critical for TFH cell differentiation via up-regulation of Bcl6 [34]. The CD28 family member ICOS is critical for the initiation and maintenance of TFH cell differentiation. ICOS deficiency in both human and mice causes severe defect in TFH cell development, GC formation and antibody production [35]. TFH cells highly express another CD28 family member, programmed cell death protein 1 (PD-1) which is a co-inhibitory molecule. PD-1 negatively regulates the number and function of TFH cells, and in turn helps prevent autoimmune diseases, such as SLE [36]. CD40-CD40 ligand (CD40L) interaction is crucial for the B cell help function of TFH cells. Defect of CD40 signaling leads to the failure of GC B cell differentiation [37]. In human, defective CD40 signaling causes hyper-IgM syndrome. These patients have decreased levels of serum IgG and IgA leading to increased susceptibility to infections.

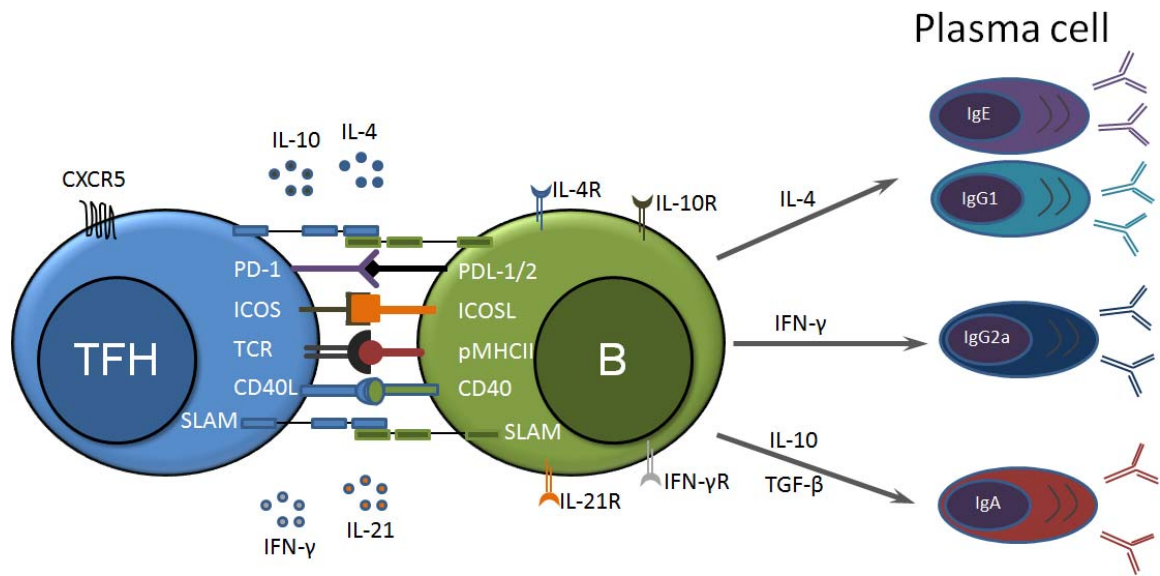


Figure 3. Delivery of the effector TFH cell function to cognate GC B cells.

This figure describes the surface molecule interactions between TFH cells and GC B cells as well as the effect of cytokines produced by TFH cells on the antibody class switching in the GC.

1.3.3: Antibody affinity maturation and class switching.

During the course of GC reaction, the affinity of BCRs to the antigens increases dramatically. This process is called antibody affinity maturation (Fig. 4) which involves two interrelated processes, clonal selection and somatic hypermutation [38].

The default fate of GC B cells is cell death via apoptosis, due to the high level of TNF receptor superfamily member 6 (Tnfrsf6, also known as Fas) expression. The survival of GC B cells requires two major signals through the BCR and CD40 (Fig. 3-4). GC B cell clones with different levels of affinity against the antigens compete with each other for the antigens captured on the FDCs and CD40 signaling from CD40L on TFH cells. The higher affinity the BCRs are, the more antigens the GC B cells internalize and present on the surface to TFH cells [39]. Therefore high-affinity GC B cells can get more “help” from TFH cells to survive, whereas low-affinity GC B cells, without TFH cell help, undergo cell death. High-affinity GC B cells subsequently undergo cell proliferation as well as somatic hypermutation of their immunoglobulin variable-region genes mediated by Activation-Induced Deaminase (AID) [39]. The mutations change the binding affinity of the resultant BCRs to the antigens. Mutated B cell clones go back and experience several cycles of further selection and somatic hypermutation. In this way, the affinity of BCRs gradually increases. When BCR affinity reaches a certain threshold, GC B cells then differentiate into plasma cells or memory B cells with the capability to produce high-affinity antibodies [40].

An antibody is a secreted form of the BCR. It consists of two heavy chains and two light chains joined to form a “Y” shaped molecule. It has two variable regions in the upper tips of the “Y” shape which give antibody the specificity of antigen binding. The constant region in the heavy chain defines the class or isotype of the antibody. There are several isotypes of antibodies including IgD, IgM, IgG, IgE and IgA. IgA and IgG each have sub-isotypes. Different antibody isotypes function in different locations and have different biological properties. For example, IgA is abundant at the mucosal areas, such as gut and respiratory tract. IgA is important for the homeostasis of the microbiota in the gut and the antiviral responses in the respiratory tract [41]. Naive B cells only express IgM and IgD on the surface. After B cell activation, B cells lose the expression of IgD and change the IgM to other isotypes through class switching. The class switching to specific isotypes can be regulated by the cytokine produced by TFH cells (Fig. 3) [42]. IL-4 can induce isotype switching to IgG1 or IgE; IFN- γ can induce isotype switching to IgG2a; IL-10 and TGF- β can induce isotype switching to IgA.

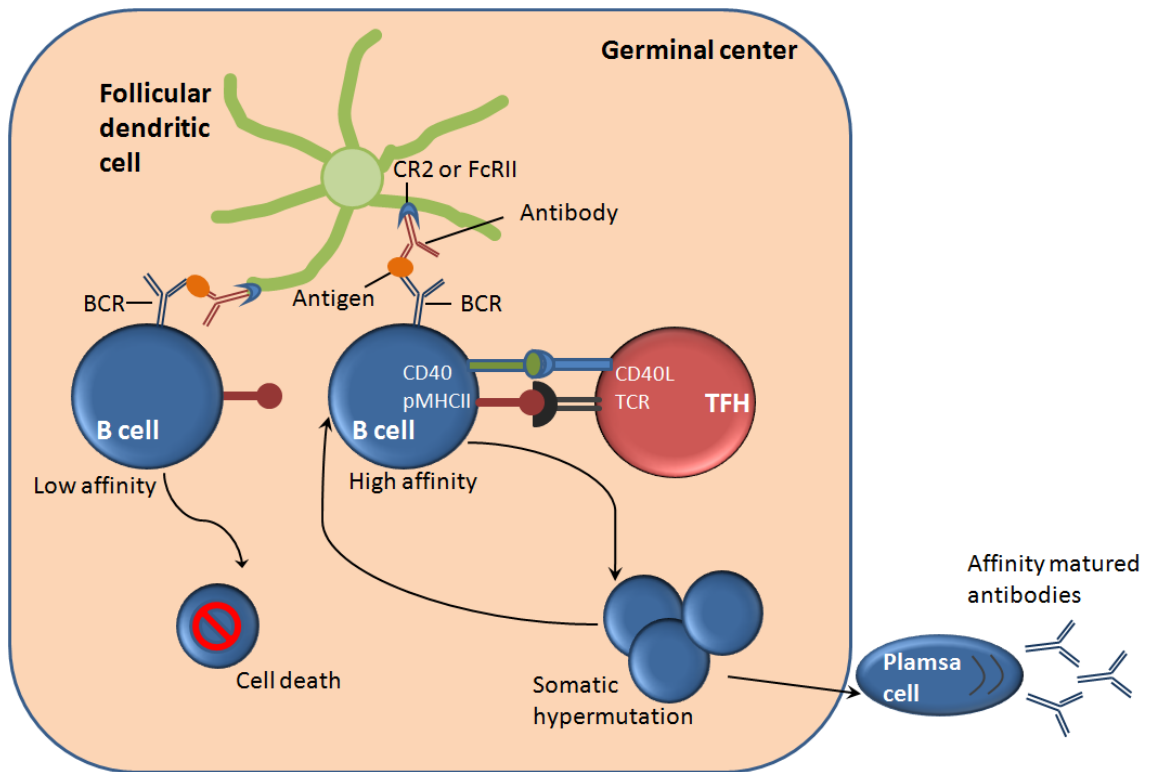


Figure 4. Affinity maturation of the antibody.

Both high- and low-affinity GC B cells bind to the antigens deposited on the FDCs that give both cells enough BCR signals to survive [39, 43]. However the number of TFH cells is limited in the GC. B cells with high-affinity BCRs capture more antigens to present on the surface. Therefore high-affinity GC B cells have an advantage over low-affinity GC B cells in the competition for the limited TFH cell help, such as CD40 signals. Selected GC B cells then undergo several rounds of proliferation, somatic hypermutation of their immunoglobulin variable-region genes and affinity selection. Finally, GC B cells exit from the GC and differentiate into plasma cells producing high-affinity antibodies. CR2, complement receptor 2; FcRII, low-affinity Fc receptor for immunoglobulin.

1.4: Bcl6 function in CD4⁺ T cells.

Bcl6 is a 95 kDa protein and a member of the BTB/POZ/zinc finger family of transcription factors [44]. The Bcl6 protein includes an N-terminal BTB/POZ domain, three PEST domains in the middle portion and six C-terminal zinc finger (ZF) DNA binding motifs (Fig. 5). It functions as a repressor of transcription. The BTB domain of Bcl6 can recruit nuclear receptor co-repressor 1 (NCOR1), NCOR2 and Bcl6 interacting co-repressor (BCOR). The middle region of Bcl6 can recruit another co-repressor, metastasis associated 1 family member 3 (MTA3) [44]. Bcl6 recruits different co-repressors to repress different target genes. The transcription repression function of Bcl6 is mainly through direct binding to the genomic locus by its ZF motifs. Alternatively, Bcl6 also can inhibit gene expression indirectly by interacting with other transcription factors, such as Tbet. In Th1 cells, Bcl6 is recruited by Tbet, which binds to the DNA, to inhibit SCOS1, SCOS3 and Tcf7 to promote Th1 cell development [45].

TFH cell differentiation requires its master transcription factor, Bcl6 [46]. Two days after T cell activation in vivo, T cells up-regulate Bcl6 and subsequently CXCR5. The up-regulation of Bcl6 requires DCs mediated antigen presentation as well as cytokines such as IL-6 and IL-12. Then they migrate to the T-B border and become TFH cells. How Bcl6 promotes TFH cell differentiation is not clear. One possible mechanism is that Bcl6 promotes gene expression through suppression of microRNAs. Bcl6 was shown to suppress mir-17~92 clusters to promote CXCR5 expression [47]. However, new evidence indicates that mir-

17~92 does not inhibit but actually induces TFH cell differentiation, which makes this microRNA cluster an unlikely target of Bcl6 suppression [48].

Bcl6 can antagonize Th1, Th2, Th9 and Th17 cell differentiation by suppressing the expression and activities of Tbet, GATA3, IL-9 and ROR γ , respectively [46, 49]. In this way, Bcl6 can ensure TFH fate during T cell differentiation. In addition, Bcl6 can suppress another transcription repressor B lymphocyte-induced maturation protein-1 (Blimp1), which is important for the differentiation of non-TFH effector T cells such as Th1 cells [50]. Blimp1 is downstream of STAT5 signaling, suppresses Bcl6 expression, and in turn inhibits TFH cell development [51].

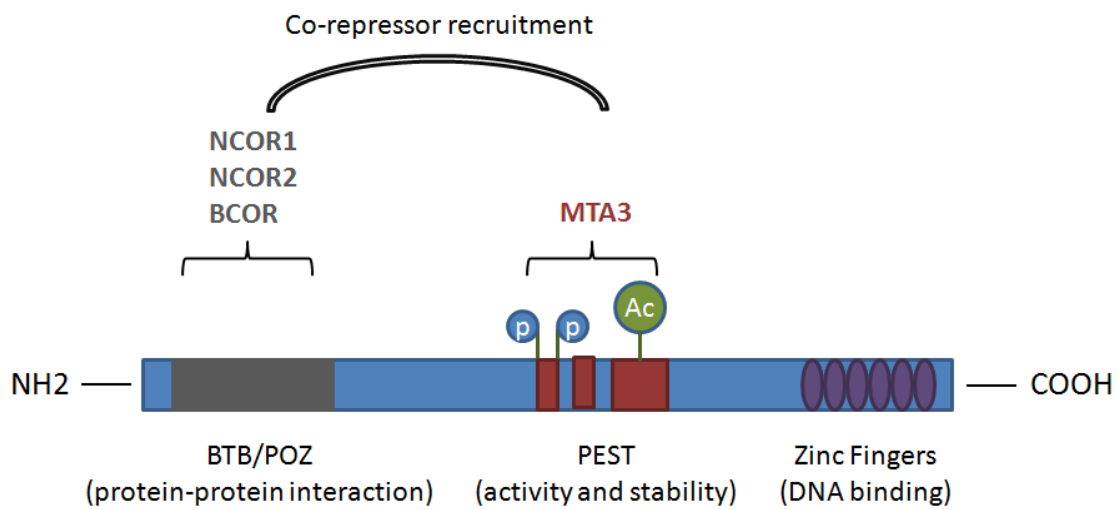


Figure 5. DNA binding and recruitment of co-repressors of the Bcl6 protein.

Schematic representation of the Bcl6 protein includes BTB/POZ domain, PEST domain and ZFs domain. The BTB/POZ domain and PEST domain are involved in the recruitment of several co-repressors. The ZFs domain gives Bcl6 the DNA binding specificity.

1.5: STAT3 function in CD4⁺ T cells.

Cytokine signaling is critical for T helper cell activation and differentiation into effector T cells. JAK (Janus kinase) /STAT signaling pathway is one of the major pathways downstream of cytokine receptor signaling. Upon cytokines binding to their own receptors, the STATs get activated and translocate into the nucleus, promoting the expression of target genes and involved in the chromatin epigenetic regulation.

In CD4⁺ T cells, STAT3 can be downstream of several cytokines, such as IL-6, IL-10, IL-21, IL-23 and IL-27 (Fig. 6) [52]. It is important for the generation of efficient cellular and humoral immune responses, as revealed from studies of STAT3-deficient human and mice. The human disease, hyper-IgE syndrome (HIES) is caused by STAT3 mutations. Those patients suffer from infectious susceptibility to fungi and virus, poor antibody responses, elevated serum IgE level, eczema and many other non-immunological defects [53].

STAT3 signaling downstream of IL-6, IL-21 and IL-23 is important for the differentiation and function of Th17 cells by inducing the transcription factors ROR γ and ROR α , as well as the effector cytokines IL-17A, IL-17F and IL-21 [18]. IL-17 cytokines are critical for the clearance of fungal infection. Due to the defect in the production of IL-17 cytokines, HIES patients are very susceptible to mucocutaneous fungal infections [53]. Elevated IgE production in HIES suggests enhanced Th2 response. However, STAT3 is required for murine Th2 cell differentiation both in vitro and vivo [54]. STAT3-deficiency reduces STAT6 binding to GATA3, cMaf and Batf which are the key transcription factors for Th2

cell differentiation. TFH cells provide help to B cells to produce high-affinity antibodies against invading pathogens. STAT3 is also critical for TFH cell differentiation [55, 56]. However, in a mouse model, STAT1 actually can compensate for the STAT3-deficiency in TFH cell differentiation [57]. Both in human and mice, STAT3-deficiency causes severe defect in the generation of effective antigen-specific antibodies [21, 55]. Since IL-4 is an important B cell help cytokine produced by TFH cells and STAT3 is required for IL-4 production in Th2 cells [54], we want to further study the role of STAT3 in the regulation of IL-4 production in TFH cells.

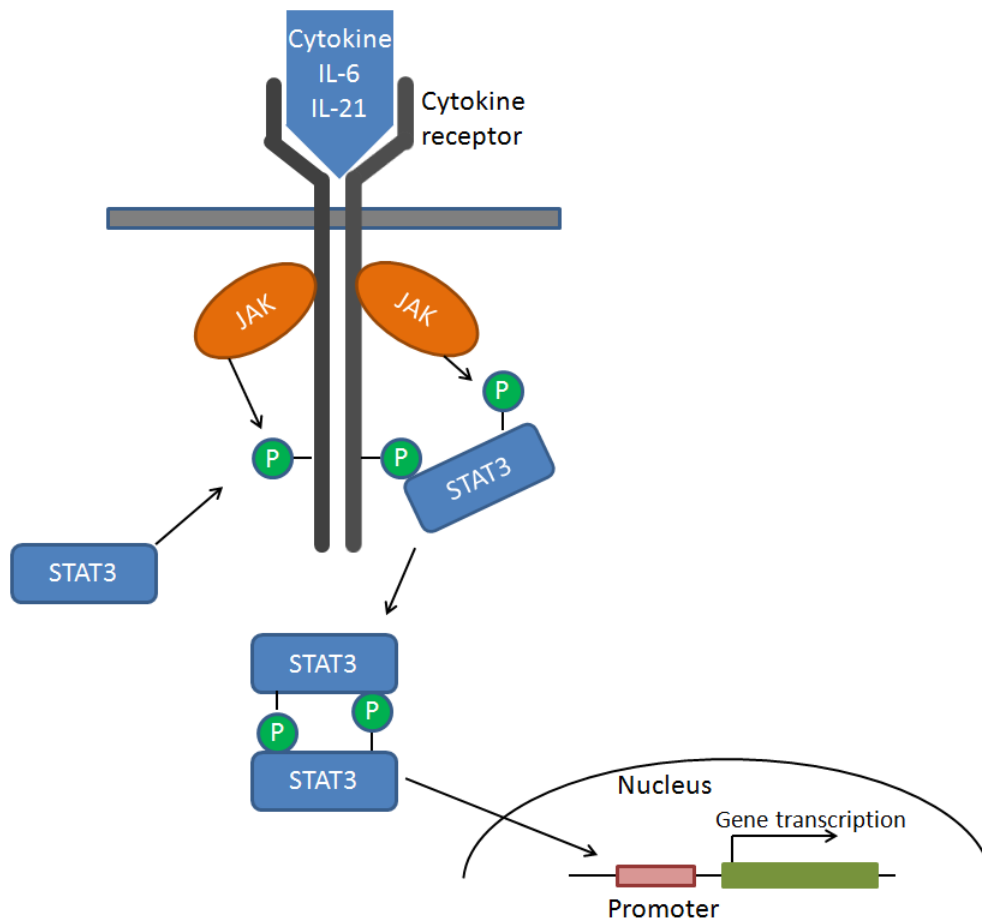


Figure 6. STAT3 signaling pathway.

Cytokines such as IL-6 and IL-21 bind to their specific cytokine receptors and activate the receptor-associated JAKs. JAKs phosphorylate the cytoplasmic tyrosine residues of the cytokine receptors which recruit STAT3. JAKs further phosphorylate recruited STAT3. Once STAT3 is phosphorylated, it dimerizes with itself or other STATs. Then the STAT3 dimer translocates into the nucleus where it binds to the consensus sites within the target promoters, promoting gene expression.

1.6: Goals of research.

IL-4 is important for the survival, proliferation and affinity maturation of GC B cells [58]. It can drive the class switching of IgG1 and IgE and is strongly implicated in atopic and allergic disease [59]. In the GC, TFH cells are the major producers of IL-4 that contribute to antibody production. The underlying molecular mechanisms of the regulation of IL-4 expression in TFH cells remain largely unknown. Stritesky et al [54] showed that STAT3 is required for the IL-4 production in Th2 cells. TFH cell differentiation and B cell help function requires STAT3 [55]. However, the role of STAT3 in IL-4 production by TFH cells is not clear and it is a question of particular interest.

Additionally, a subpopulation of Treg cells in the GC was discovered that appear to act as suppressors of TFH and GC B cells [60-62]. Besides expressing Foxp3, these regulatory follicular T cells or “TFR” cells express CXCR5, ICOS, and PD-1, and depend on Bcl6 for differentiation and localization to the B cell follicle. In vitro and in vivo studies have shown that TFR cells can suppress TFH- and GC B-cell proliferation and can regulate GC B cell differentiation as well [60-64].

However how TFR cells affect antibody production is still unresolved. Several studies have shown that TFR cells repress antibody production [60-64]. Furthermore, Linterman et al demonstrated that TFR cells control the outgrowth of non-antigen-specific GC B cells and helped maintain high titers of high-affinity antigen-specific antibodies [61]. Therefore we used two mouse models, Bcl6

Treg conditional knockout mice and STAT3 Treg conditional knockout mice, to study the precise mechanisms by which TFR cells control antibody responses.

Chapter 2 - An inhibitory role for the transcription factor STAT3 in controlling IL-4 and Bcl6 expression in follicular helper T cells

2.1: Introduction.

In the course of an immune response, CD4⁺ T helper cells differentiate into unique effector lineages that promote different immune responses via the secretion of specialized effector cytokines. TFH cells are a CD4⁺ T cell lineage whose identified function is to promote formation of GCs and select B cell clones that produce high-affinity antibodies (reviewed in [42, 65-68]). TFH cells are typically identified as CD4⁺ CXCR5⁺ and PD-1^{high} T cells, and have an activated T cell phenotype but are CD25^{neg}. TFH cells control the outcome of the GC B cell response, and are critical for memory B cell and plasma cell development. TFH cells produce IL-21, a cytokine that potently promotes B cell activation and antibody secretion. While TFH cells are required for the production of high-affinity antibodies, excessive numbers of TFH cells can promote autoimmunity by helping B cells produce self-reactive antibodies [69-71]. The proper regulation of TFH cell differentiation is therefore essential for effective antibody responses and preventing development of autoimmune disease.

The Bcl6 transcriptional repressor protein is up-regulated in TFH cells and is considered a master regulator for the TFH subset [47, 72, 73]. STAT factors are upstream of Bcl6 in TFH cell differentiation and receive cytokine signals to bind to the Bcl6 promoter and induce high levels of Bcl6 expression. Specifically, activated CD4 T cells exposed to the cytokines IL-6, IL-21 and IL-12 activate

STAT3 and STAT4 to promote TFH cell differentiation via up-regulation of Bcl6 [42, 65, 74-77]. STAT1, activated by IL-6 or type I IFN can also promote Bcl6 transcription and TFH cell differentiation [78, 79], although in certain contexts, type I IFN can inhibit TFH cell differentiation [56].

Although STAT3 is not required for early T cell development [80, 81], STAT3 plays many important roles in T cell immune responses, particularly in the development of the pro-inflammatory Th17 cells [82-86] and formation of T cell memory [87, 88]. Furthermore, STAT3 is required for Th17-mediated colitis, as well as regulatory control of colitis [89, 90]. Mutations in STAT3 can lead to disease termed HIES, which is characterized by elevated IgE, repeated infections, chronic dermatitis and lack of Th17 cells [91]. HIES has a complex pathology and aspects of the disease appear to be mediated by non-lymphoid and even non-hematopoietic cells [92].

Two previous studies on the role of STAT3 in TFH cell differentiation following lymphocytic choriomeningitis virus (LCMV) infection revealed that TFH cell development was not strictly dependent on STAT3 function [56, 79]. Thus, LCMV induced a delayed [79] or weakened [56] TFH cell response in CD4-cre STAT3^{fl/fl} conditional KO mice, where STAT3 was deleted specifically in T cells. Ray et al [56] revealed that part of the defect in TFH cell development in the LCMV system was due to the failure to properly up-regulate Bcl6 in the absence of STAT3, a defect that was partially due to heightened sensitivity to Type I IFN signaling in STAT3-deficient CD4 T cells [56]. Additionally, STAT3-deficient CD4 T cells responding to LCMV developed a strong Th1 phenotype [56], indicating

that STAT3 has an important role in repressing Th1 cell development. However, a major question unaddressed by these studies was whether the findings observed for STAT3 and TFH cells in a LCMV infection model were broadly applicable to the role of STAT3 in TFH cell responses in other immune contexts. We therefore used CD4-cre STAT3^{fl/fl} conditional knockout mice to examine TFH cell differentiation in the gut immune response, in PPs, as well as with immunization with a strong inducer of the germinal center response, SRBC. Our data contrasts with the Ray et al study on STAT3 in TFH cells on the control of Bcl6 and IL-4 expression by STAT3 [56], and thus provides important new insights into the role of STAT3 in Bcl6 expression and TFH cell differentiation.

2.2: Materials and Methods.

Mice and immunizations. STAT3^{fl/fl} mice [56] were backcrossed to CD4-cre transgenic mice and the C57BL/6 strain for at least six generations [54]. TCR-transgenic OT-II mice and B6.SJL-Prp^{rc}^aPep^c^b/BoyJ (BoyJ) mice were purchased from The Jackson Laboratory (JAX). Control mice for CD4-cre STAT3^{fl/fl} (STAT3KO) mice were litter-mate STAT3^{fl/fl} (WT) mice. Mice were bred under specific pathogen-free conditions at the laboratory animal facility at IUSM and were handled according to protocols approved by the IUSM Animal Care and Use Committee. For SRBC immunization, mice were intraperitoneally (i.p.) injected with 1 x 10⁹ SRBCs (Rockland Immunochemicals) and were sacrificed at the indicated day. For Ovalbumin (OVA; Sigma) immunization, 50 ug OVA was mixed with Imject Alum (Pierce), and the mixture was injected i.p.

Cell culture and retrovirus transduction. CD4⁺CD62L⁺ WT and STAT3KO OT-II T cells were isolated with the CD4⁺CD62L⁺ T cell isolation kit II (Miltenyi), then were activated with 1ug/ml OVA peptide (Anaspec) and spleen-derived APCs for 3 days (one round) or 5 additional days (two rounds) under Th2 culture conditions: 40ng/ml IL-4 (Peprotech), 10ug/ml anti-IFN- γ and anti-IL-12 (BioXCell). After 3 or 8 days of culture, cells were collected for ICS as described below. For retroviral transduction, CD4⁺CD62L⁺ WT and STAT3KO T cells were activated with 1ug/ml anti-CD3, 2 ug/ml anti-CD28 and splenic APCs under Th2 condition. At 40h, cells were spin infected with control H2K^k, Bcl6-H2K^k-expressing retroviruses. After spin infection, supernatant was removed and fresh Th2 condition medium with 10 U/ml rhIL-2 were added back to cell culture. After 3 days, cells were collected for ICS as described below.

Flow cytometry reagents. Anti-CXCR5 (2G8), anti-CD95 (Jo2), GL7 (GL7), anti-active Caspase3 (C92-605), anti-IL-4 (11B11) and anti-Bcl6 (K112-91) antibodies were from BD Biosciences. Fixable viability dye, AnnexinV detection kit, and anti-CD38, anti-IL-13 (eBio13A), anti-IL-21 (mhalx21), anti-GATA3 (TWAJ), and anti-Foxp3 (FJK-16s) antibodies were from eBioscience. Anti-CD4 (GK1.5), anti-B220 (RA3-6B2), anti-IgG1 (RMG1-1), anti-CD45.1 (A20), anti-PD-1 (29F.1A12), anti-IL-10 (JES5-16E3), anti-interferon- γ (XMG1.2) were from Biolegend.

Cell staining for flow cytometry. After red blood cell lysis, total spleen cells were incubated with anti-mouse CD16/CD32 (BioXcell) for 5 minutes at room temperature, followed by surface staining for the indicated markers. For

intracellular transcription factor staining, after surface markers were stained, cells were fixed and stained with antibodies against transcription factors by following Foxp3 fixation kit (eBioscience) instructions. Cell events were collected on an LSRII flow cytometer (Becton Dickinson).

Intracellular cytokine staining. Cells were stimulated with PMA (75ng/mL) plus ionomycin (1ug/mL) for 5 hours in DMEM cell culture medium, then fixed and stained for indicated cytokines as described [93]. Golgistop and Golgiplug (BD Biosciences) were used during the stimulation to inhibit cytokine secretion.

Adoptive cell transfers and immunization. CD4⁺CD62L⁺ MACS-purified WT and STAT3KO OT-II T cells were labeled with 5 μ M Cell Tracer Violet (Life Technologies). 5×10^5 cells were injected i.v. per BoyJ recipient mice. After 18 hours, 50 ug Ovalbumin (Sigma) mixed with Imject Alum (Pierce) was injected i.p. into recipient BoyJ mice. CD45.1⁻ OT-II cells from spleen were analyzed by flow cytometry for CXCR5, PD1, Bcl6, Tbet and GATA3.

Peyers patch isolation. PPs were cut using scissors from the small intestine and incubated for 10 mins at 37 °C in PBS containing 1% FBS, 4mM EDTA and 15 mM HEPES (PH 7.2). PPs were washed twice, with vigorous vortexing before spinning, in PBS. Soluble cell debris in supernatants were removed after centrifugation. Isolated PPs were broken apart between two frosted glass microscope slides to generate single cell suspension for flow cytometry staining or intracellular staining (ICS) described above.

Bone marrow chimeras. Recipient CD45.1⁺ BoyJ mice were lethally irradiated with 1,100 Rad and reconstituted with 2x10⁶ mixture of nucleated bone marrow (BM) cells from either CD45.2⁺ WT mice plus CD45.1⁺ BoyJ mice, or CD45.2⁺ STAT3KO mice plus CD45.1⁺ BoyJ mice by i.v. injection. Thus, WT chimeras were used as a control to compare the repopulation of WT CD45.2⁺ BM with the repopulation by STAT3KO CD45.2⁺ BM. This system has been described previously [47]. Chimeric mice were immunized with SRBC approximately 12 weeks after reconstitution.

Antibody titer analysis. Antibody titers of SRBC-specific in serum were measured by ELISA, as previously reported [93]. Briefly, 96 well Nunc-Immuno plates (Sigma) were coated with SRBC membrane protein overnight at 4 °C. Wells were blocked with 10% FCS and diluted serum was added and incubated at room temperature for 2 h. A peroxidase-conjugated anti-mouse IgG1 (BD), anti-mouse IgG or anti-mouse IgM antibodies (Sigma) were used as secondary antibody.

Statistical Analysis. All data analysis was done using Prism Graphpad software. Unless otherwise stated, Student t test or ANOVA with Tukey post hoc analysis were used. Only significant differences ($p < 0.05$) are indicated in figures.

2.3: Results.

2.3.1: TFH cell development in the PPs can occur independent of STAT3, and STAT3 regulates the cytokine response of TFH cells.

To analyze the role of STAT3 in TFH cell development, we obtained conditional knockout mice in which STAT3 is deleted from T cells via a CD4-cre transgene (CD4-cre STAT3^{fl/fl} mice, termed STAT3KO mice) [54]. Initially, we analyzed the TFH cell and GC B cell responses that occur in the intestinal PPs, to determine if STAT3 in T cells was essential for normal homeostatic TFH cell responses and the related GC B responses in the PP (Fig. 7). The percentage and overall numbers of CD4⁺ T cells in PP were decreased significantly in the STAT3KO mice (Fig. 7B-C). As shown in Fig. 7D, the loss of STAT3 resulted in 40% inhibition of the proportion of TFH cells developed from the CD4⁺ T cell compartment in the PP, and the absolute number of PP TFH cells in STAT3KO mice was about one third of the number in WT PP (Fig. 7E). However, the loss of STAT3 in T cells did not significantly affect the development of PP GC B cells (Fig. 7G). Loss of STAT3 in T cells did affect the rate of GC B cells in the PP switching to IgG1, as dramatically increased percentages of IgG1⁺ GC B cells were seen in the PP of STAT3KO mice (Fig. 7I-K). This result was confirmed by ELISPOT assays showing significantly increased numbers of IgG1-producing cells in the PP of STAT3KO mice (Fig. 7L).

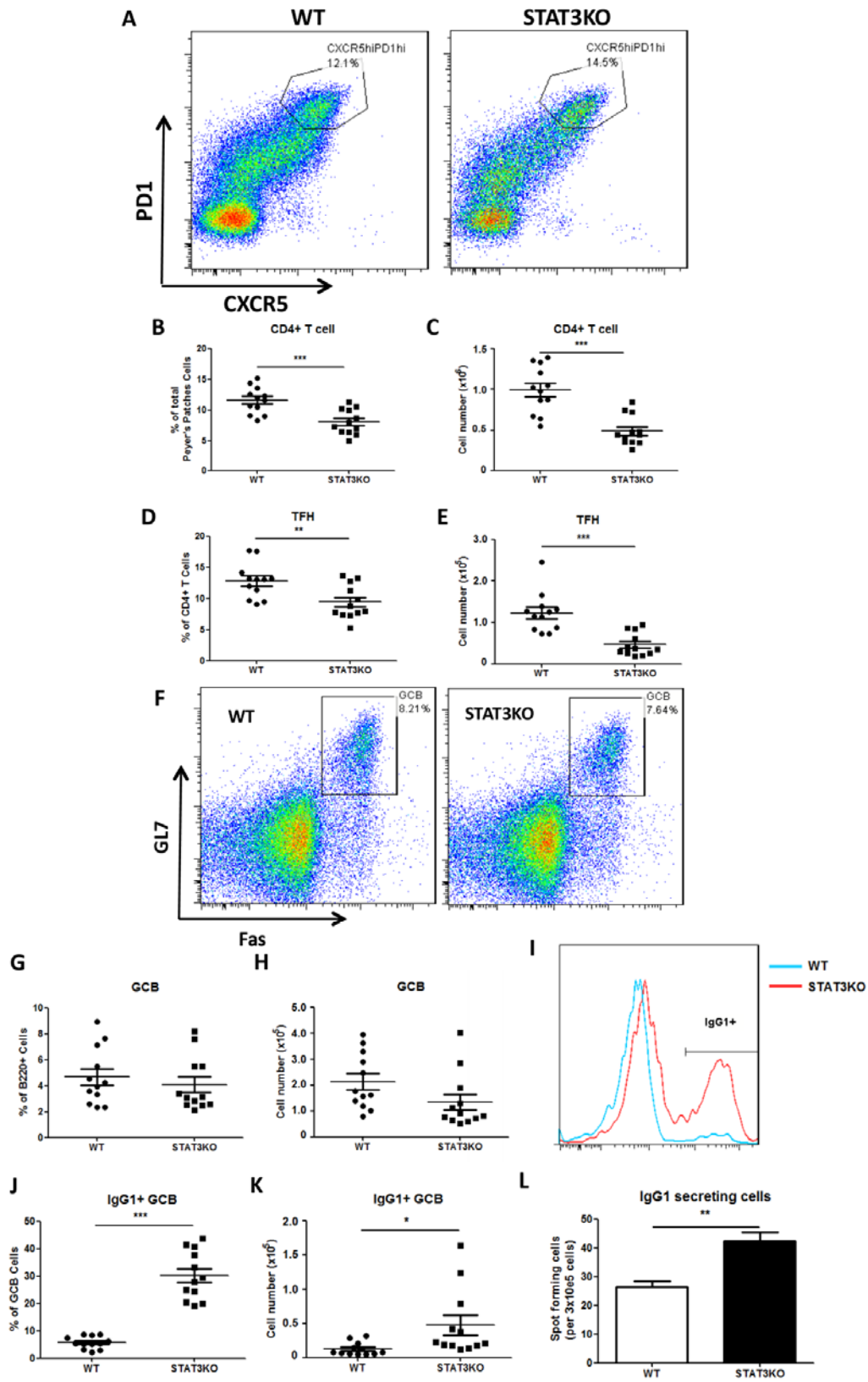


Figure 7. STAT3 is not required for normal TFH cell or GC B cell frequencies in the Peyer's patches, but regulates IgG1 class switching.

(A) TFH cells are gated on Foxp3⁻CXCR5^{hi}PD1^{hi} cells within the CD4⁺ T cell population, examples from PP of STAT3^{fl/fl} (WT) and CD4-cre STAT3^{fl/fl} (STAT3KO) mice are shown. Percentages (B) and absolute numbers (C) of CD4 cells within the PPs. (D) Average PP TFH cell percentages within the CD4⁺ T cell population in WT and STAT3KO mice. (E) Absolute numbers of PP TFH cells in WT and STAT3KO mice. (F) GC B cells are gated on Fas⁺GL7⁺ cells within the B220⁺ population, examples from PP of WT and STAT3KO mice are shown. Average PP GC B cell populations by percentage (G) and absolute number (H). (I) Histogram showing IgG1⁺ GC B cells from WT and STAT3KO, gated on the GC B population. Average IgG1⁺ GC B cell populations in terms of percentage (J) and absolute number (K). (L) IgG1 secreting cells in 3x10⁵ WT and STAT3KO total PP cells, assayed by ELISPOT. (n=3, mean ± SEM). Each symbol represents one mouse (n=12, mean ± SEM). Data are combined from four independent experiments, except for ELISPOT, which was repeated once. *p <0.05, **p <0.01, ***p <0.001 by t-test.

We next wondered what could account for the increased percentages of IgG1⁺ GC B cells in STAT3KO mice and decided to examine cytokine production by PP TFH cells. As shown in Fig. 8A-B, PD1^{hi} Bcl6^{hi} T cells (TFH cells) from STAT3KO mice PP produce markedly higher percentages of IFN- γ - and IL-4-positive cells as detected by ICS. Representative ICS cytokine flow plots are shown in Fig. 9. As expected for loss of STAT3 activity, IL-21 was significantly lower in STAT3KO PP TFH cells (Fig. 8B). Non-TFH cells had much lower frequencies of cytokine producing cells (Fig. 8C), and STAT3KO PP non-TFH cells expressed more IFN- γ , IL-10 and less IL-21. However IL-4 was not increased in the STAT3KO non-TFH cells (Fig. 8C), indicating a unique regulation of IL-4 in TFH cells. Notably, whereas WT PP TFH cells had almost no double IFN- γ /IL-4-expressing cells or double IFN- γ /IL-10-expressing cells, the STAT3KO PP TFH cells had far higher levels of these double cytokine-expressing cells (Fig. 8D-E). These results indicate that STAT3 regulates expression of cytokines by TFH cells, and inhibits dual expression of cytokines. To further confirm these results and gain insight into the mechanism for increased IL-4 expression, we sorted WT and STAT3KO PP TFH cells by FACS, made RNA and cDNA directly from the purified cells and tested gene expression by Quantitative RT-PCR (QPCR). We found as expected, that in contrast to non-TFH cells, TFH cells constitutively express Il4 (Fig. 10A). We found 3-fold higher levels of Il4 and the master transcription factor for Th2 cells, GATA3, in the absence of STAT3 (Fig. 10A-B). We also observed significant

increases in the levels of Bcl6 and Prdm1 (Blimp1) in the STAT3KO PP TFH cells (Fig. 10C-D).

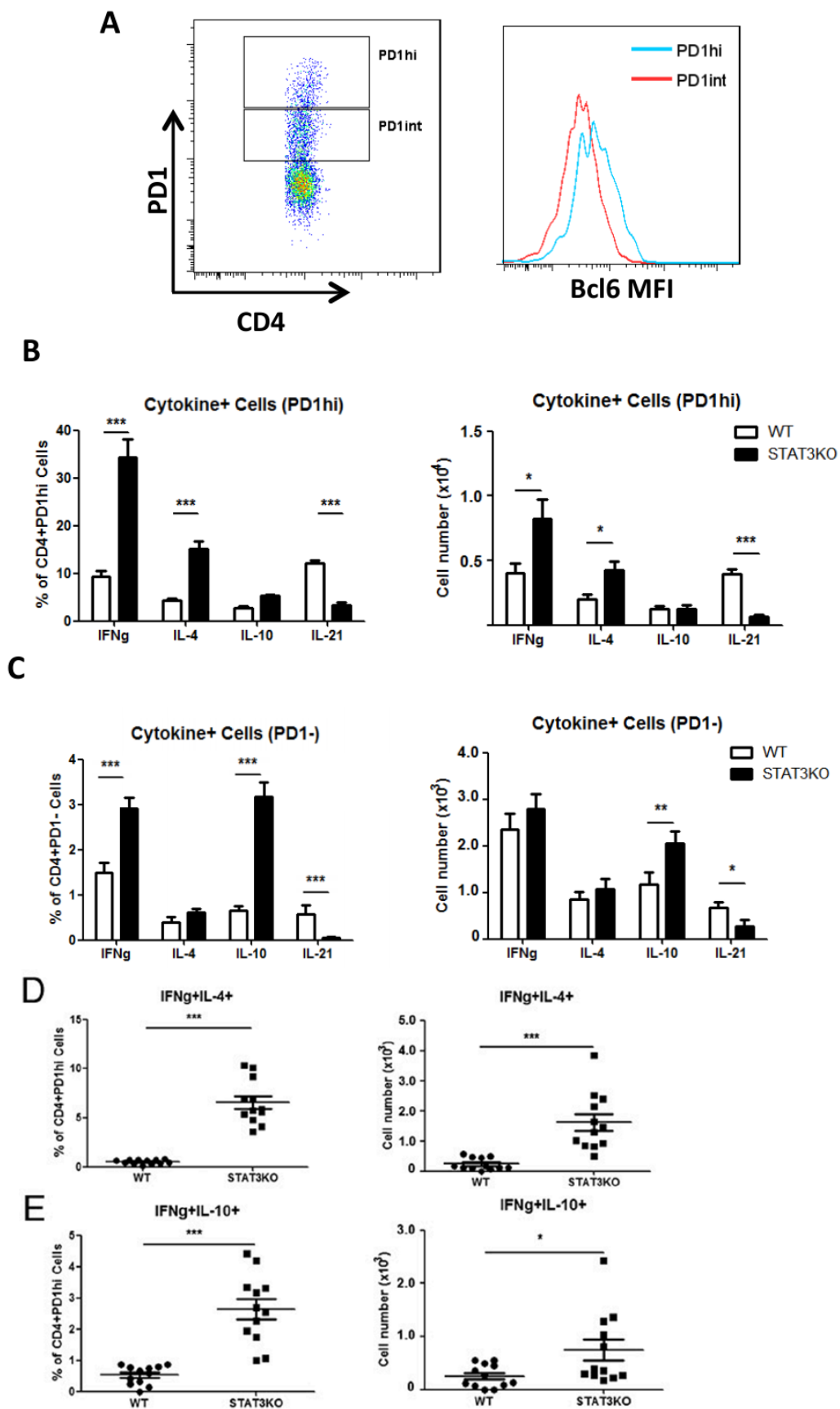


Figure 8. Deregulated cytokine production in STAT3-deficient TFH cells in the PP.

(A) PD1^{hi} and PD1^{int} CD4⁺ cell gates and increased Bcl6 mean fluorescent intensity (MFI) on the PD1^{hi} population. Fixation for ICS degrades CXCR5 staining and thus for ICS, TFH cells are defined as CD4⁺PD1^{hi}. Average percentage and absolute number of cytokine-producing (IFN- γ ⁺, IL-4⁺, IL-10⁺ and IL-21⁺) in CD4⁺PD1^{hi} TFH (B) and CD4⁺PD1^{neg} (C) cells from WT and STAT3KO PP measured by ICS (n=4, mean \pm SEM). Average percentage and absolute number of double cytokine-producing (D) IFN- γ ⁺IL-4⁺ and (E) IFN- γ ⁺IL-10⁺ populations in PD1^{hi}CD4⁺ T cells. Each symbol represents one mouse (n=12, mean \pm SEM). Data are combined from four independent experiments. *p <0.05, **p <0.01, ***p <0.001 by ANOVA (B-C) or t-test (D-E).

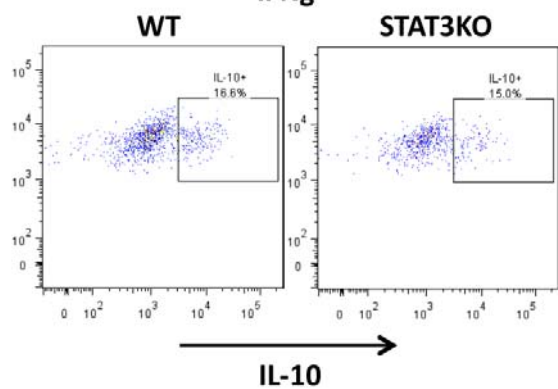
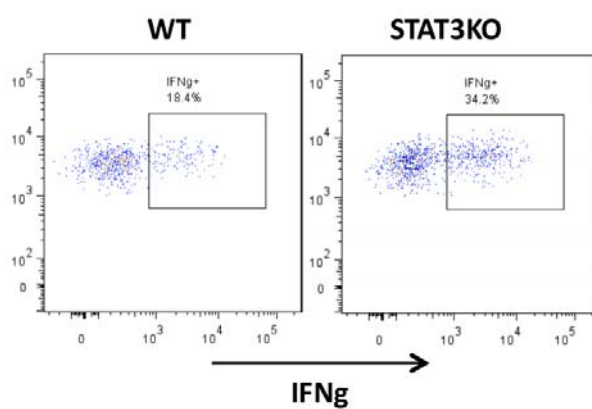
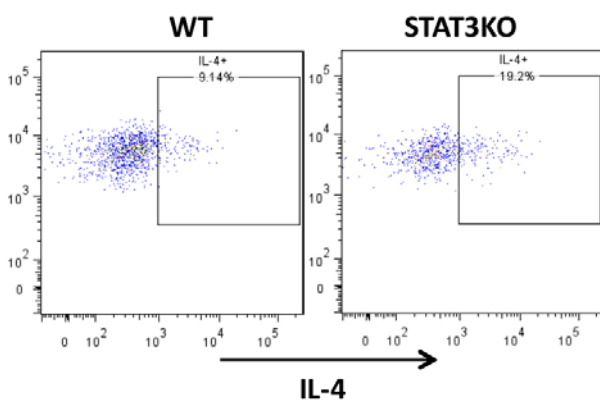
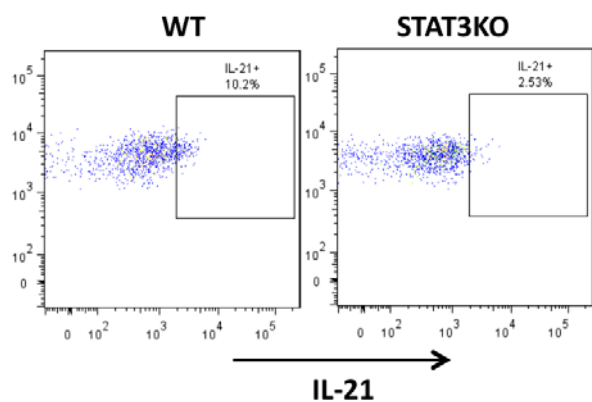


Figure 9. Examples of cytokine staining and gates showing staining controls.

Seven days after SRBC immunization, spleen cells were stimulated with PMA plus ionomycin and stained intracellularly for the indicated cytokines. Plots are gated on CD4⁺ T cells. Gated regions show cytokine positive cells. Isotype controls for each antibody were used to set gates; for isotype controls, 0-1% of the cells were in the gated region for all cytokines analyzed.

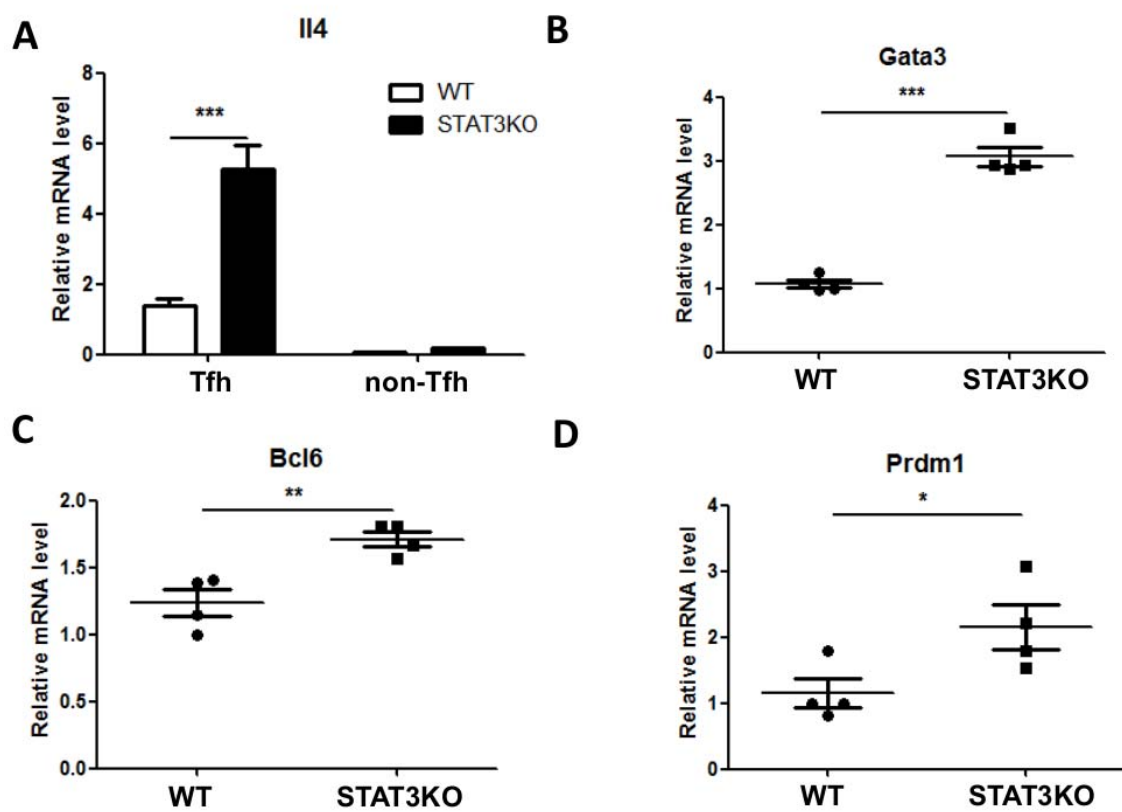


Figure 10. Altered gene expression in STAT3-deficient TFH cells in the PP.

Relative mRNA expression was determined by QPCR. (A) Il4 gene expression from freshly-isolated PP TFH and non-TFH cells (CXCR5-PD1-) from WT and STAT3KO mice (n=4, mean \pm SEM). *p <0.05 (ANOVA). (B-D) Bcl6, Prdm1 and Gata3 gene expression in freshly-isolated PP TFH cells. TFH cells were gated for flow cytometry as in Fig. 7. Each symbol represents one mouse (n=4, mean \pm SEM). *p <0.05, **p <0.01, ***p <0.001 (t-test).

2.3.2: STAT3 is required for acute TFH cell development in the spleen and represses IL-4 expression by TFH cells.

In order to test if our results from analyzing STAT3 function in PP TFH cells were applicable to other TFH cell responses, we immunized mice with SRBC, a potent inducer of GC B cell and TFH cell responses. Like CD4⁺ T cells in the PP, the total number of CD4 T cells was also decreased in the spleen of STAT3KO mice (data not shown). As shown in Fig. 11, STAT3 was required for development of a normal TFH cell response in the spleen, particularly in the early stage of the reaction, analogous to what was previously reported in LCMV infection [79]. Thus, at 3 days post-immunization, TFH cells in STAT3KO spleens were 10-fold lower than WT (Fig. 11A-C), but by day 7, the response of the STAT3-deficient TFH cell response increased such that it trended lower but was not significantly different (Fig. 11D-F). We also analyzed Bcl6 expression by intracellular staining and found that as in the PP TFH cells, loss of STAT3 led to significantly higher Bcl6 expression specifically in TFH cells (Fig. 11G-H). However, in contrast to the PP, the loss of STAT3 strongly impacted GC B cell and GC B IgG1⁺ cell development in the spleen: both responses were impaired at the early time-point and never developed above the WT level in the STAT3KO (Fig. 12A-D). Consistent with a decreased GC B cell reaction, anti-SRBC IgG titers were several fold lower in the STAT3KO, while anti-SRBC IgM levels were not affected (Fig. 12E-G). We next examined cytokine production by splenic STAT3KO TFH cells after SRBC immunization, and compared to WT TFH cells, found increased IL-4 and decreased IL-21 consistent with the PP TFH cells.

However in contrast to the PP TFH cells, IFN- γ was decreased in STAT3KO TFH cells (Fig. 13A). As in PP, the increase in IL-4 was specific to the STAT3KO TFH cells, and was not observed in STAT3KO non-TFH cells (Fig. 13B). As in the PP TFH cells, dual cytokine-expressing cells were significantly increased in the spleen TFH cell population (Fig. 13C-D).

2.3.3: Intrinsic effects of STAT3 on TFH cell function.

To confirm and extend our results, and test if the alterations in cytokine expression were an intrinsic effect of STAT3-deficiency on CD4 T cells, we utilized the OTII TCR transgenic system, where we generated both OTII TCR⁺ STAT3^{fl/fl} mice and OTII TCR⁺ CD4-cre STAT3^{fl/fl} mice. These OVA-specific CD4 T cells were transferred to congenic recipients, immunized with OVA-Alum, and donor TFH cell cytokine production was assayed by flow cytometry (Fig. 13E-F). In this system, STAT3-deficient T cells produced TFH cells, but at a significantly lower percentage than control T cells (Fig. 14A). Notably and consistent with our earlier results, the STAT3-deficient TFH cells produced significantly more IL-4, and also had an increase in double cytokine-expressing cells. The OVA-specific STAT3-deficient TFH cells also expressed higher levels of GATA3 and Bcl6 as well as Tbet (Fig. 15B-D), consistent with the STAT3-deficient TFH cells in the PP. Thus, STAT3 plays a critical role in controlling IL-4 expression in TFH cells and is intrinsically required for the de novo generation of TFH cells in an acute antigen challenge environment.

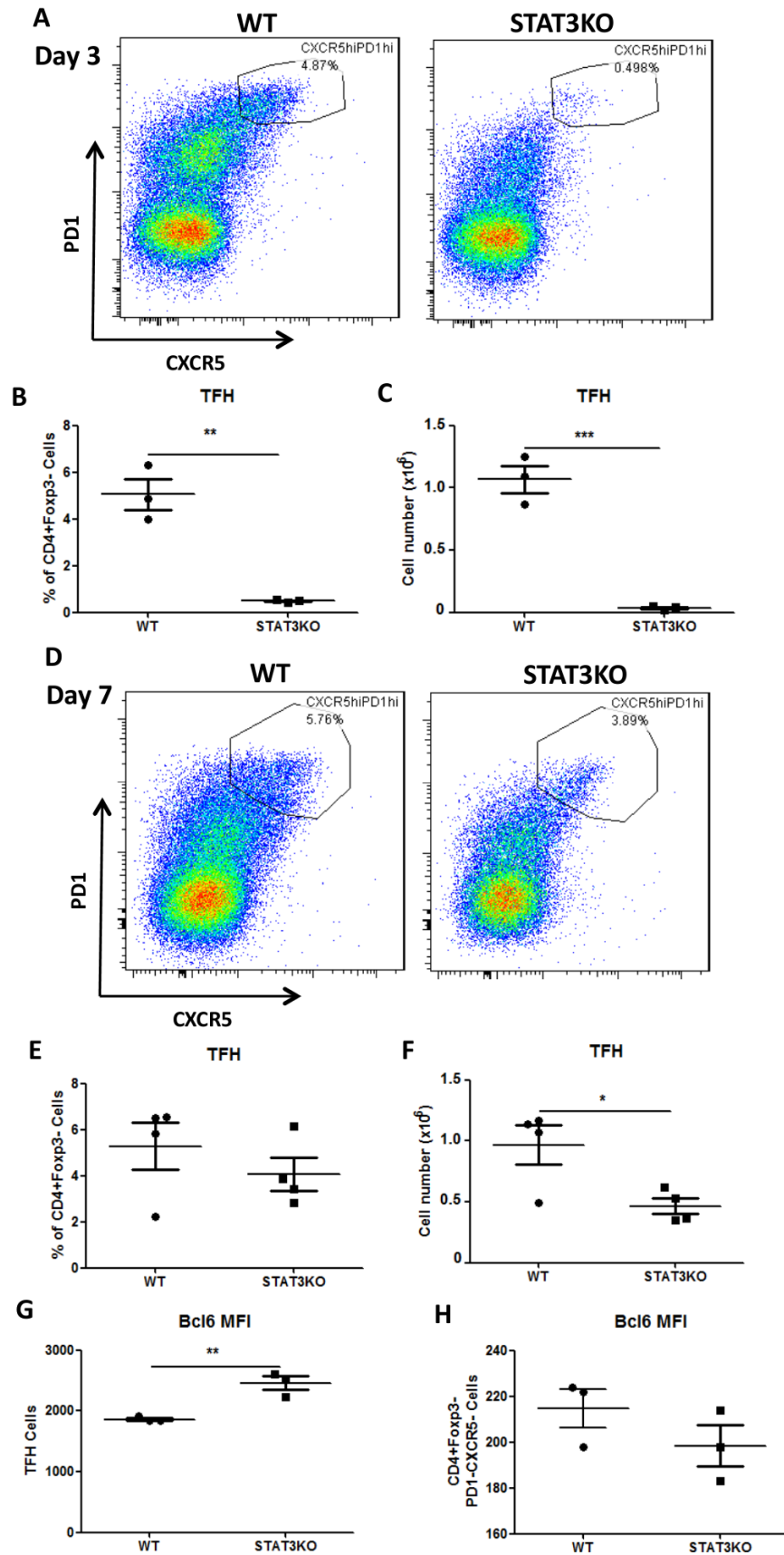


Figure 11. Delayed TFH cell responses in the spleen of STAT3KO mice after immunization.

(A) TFH cells are gated on $CD4^{+}Foxp3^{-}CXCR5^{hi}PD1^{hi}$ cells. Average TFH cell percentages (B) and absolute numbers (C) in WT and STAT3KO mice (n=4, mean \pm SEM), 3 days post-immunization (dpi) with SRBC. D) Day 7 TFH cells are gated as in (A). Average TFH cell percentages (E) and absolute numbers (F) in WT and STAT3KO mice (n=4, mean \pm SEM), 7 dpi with SRBC. G) Bcl6 MFI of TFH cells at 7 dpi (n=3, mean \pm SEM). H) Bcl6 MFI of non-TFH cells at 7 dpi (n=3, mean \pm SEM). **p <0.01, ***p <0.001 (t-test). Each symbol in graphs represents one mouse. Data are representative of two independent experiments with similar results.

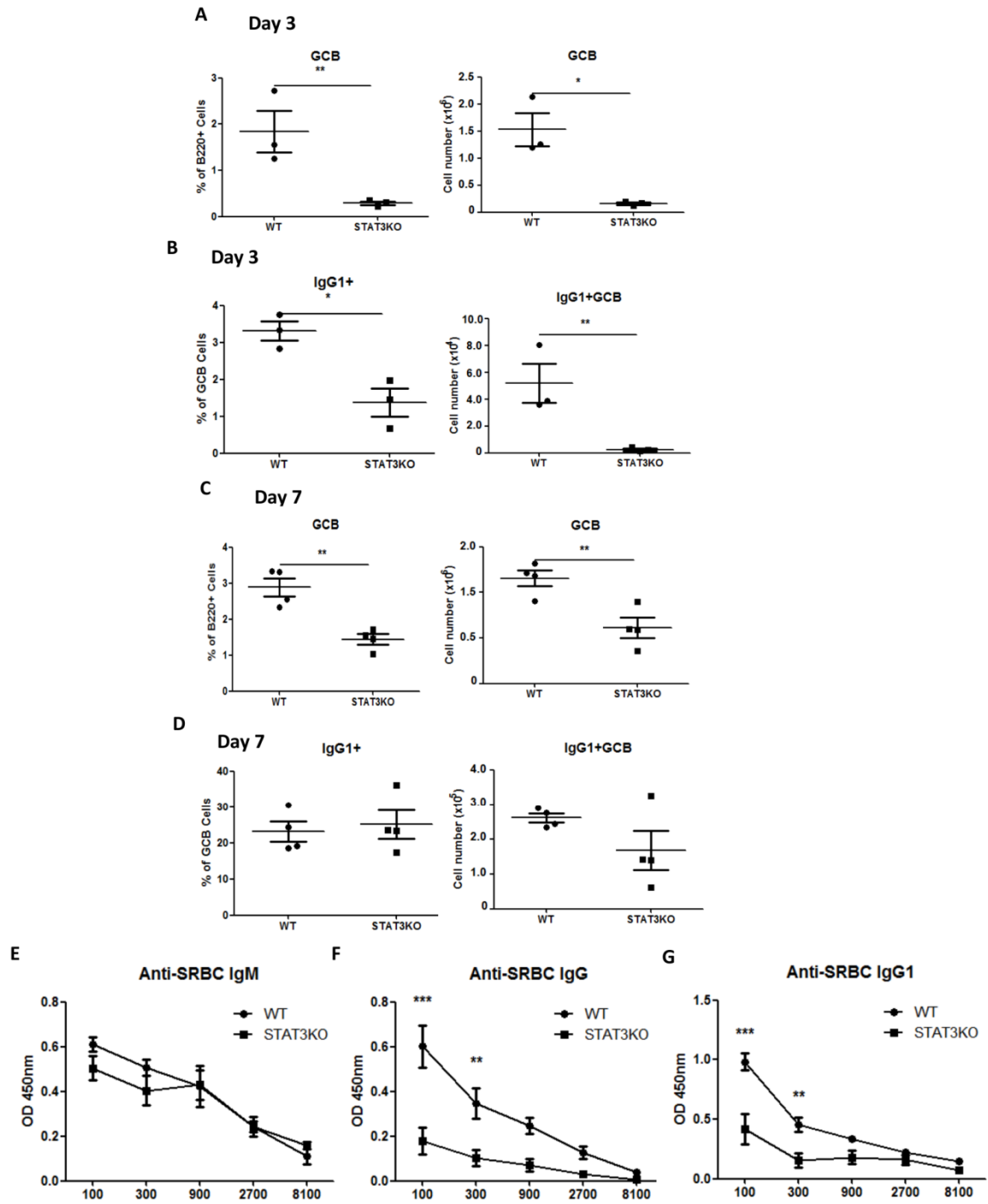


Figure 12. Weak GC B cell responses and antibody responses following SRBC immunization when STAT3 is absent in T cell.

Mice were immunized with SRBC and spleen analyzed 3 or 7 dpi. (A, C) GC B cells and (B, D) IgG1⁺ GC B cells were gated as in Fig. 7. Average GC B cell populations in terms of percentage in B220⁺ cells and absolute number at 3 dpi (A, B) and 7 dpi (C, D). Each symbol represents one mouse (n=3-4, mean \pm SEM). *p <0.05, **p <0.01 (t-test). Data are representative of two independent experiments with similar results. (E-G) Anti-SRBC antibody responses measured by ELISA at 7 dpi. (E) anti-SRBC IgM, (F) anti-SRBC IgG and (G) anti-SRBC IgG1 titers (n=4, mean \pm SEM). *p <0.05, **p <0.01, ***p <0.001.

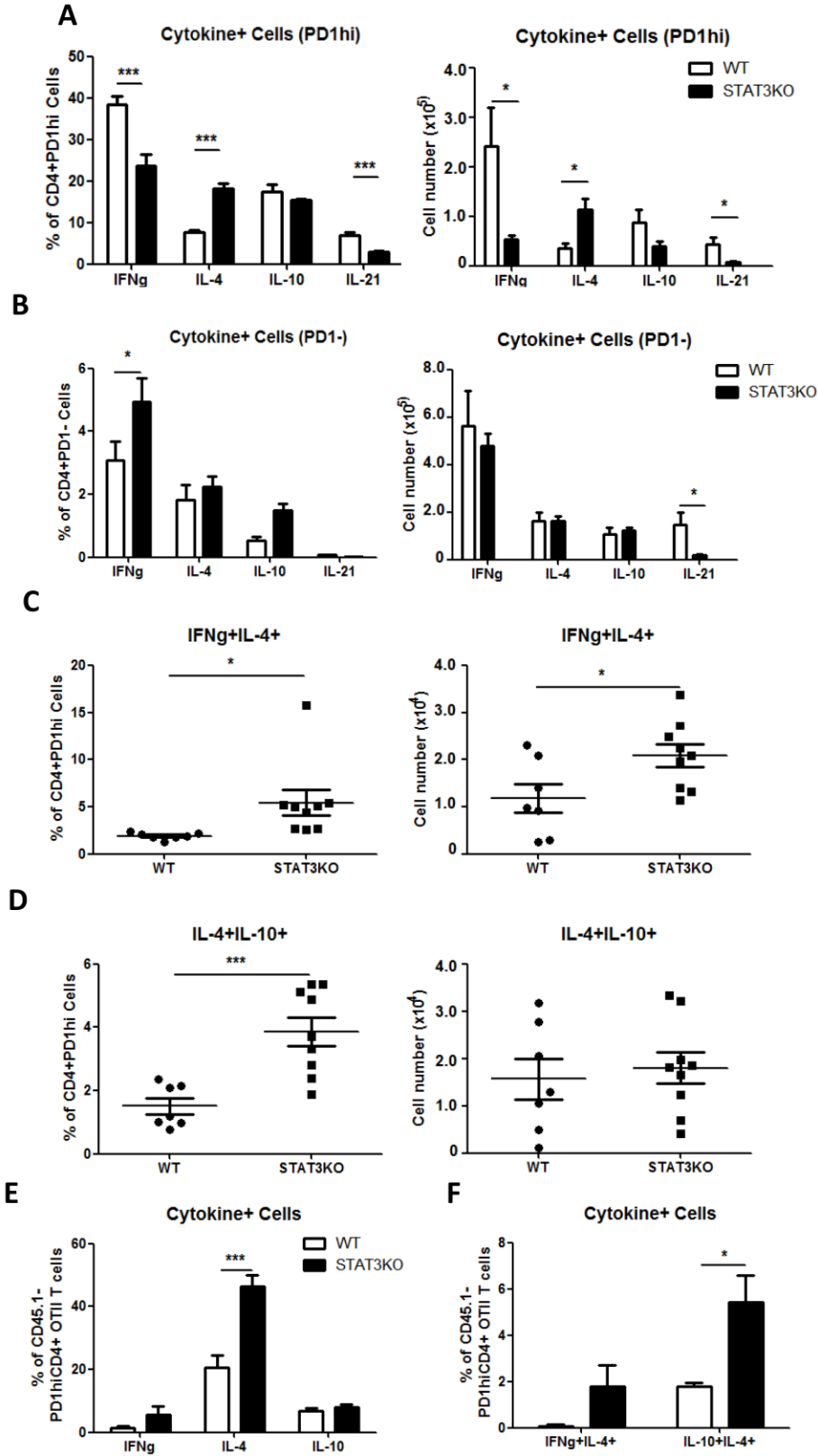


Figure 13. STAT3 controls IL-4 expression in TFH cells under multiple conditions.

Cytokines expressed by TFH cells were measured by ICS and TFH cells were gated as in Fig. 8. (A) Average percentage and absolute number of cytokine-producing (IFN- γ ⁺, IL-4⁺, IL-10⁺ and IL-21⁺) TFH cells in the spleens of WT and STAT3KO mice 7 dpi with SRBC (n=4-6, mean \pm SEM). (B) Average percentage and absolute number of cytokine-producing (IFN- γ ⁺, IL-4⁺, IL-10⁺ and IL-21⁺) non-TFH cells in the spleens of WT and STAT3KO mice 7 dpi with SRBC (n=4-6, mean \pm SEM). Average percentage and absolute number of double cytokine-producing IFN- γ ⁺IL-4⁺ (C) and IL-10⁺IL-4⁺ (D) cells within the populations assayed in (A). Each symbol represents one mouse (n=4-6, mean \pm SEM). (E-F) WT and STAT3KO OTII TCR transgenic CD45.2⁺CD4⁺ T cells were transferred to CD45.1⁺ BoyJ mice, and the recipient BoyJ mice were immunized with OVA-Alum. TFH cells were analyzed for cytokine expression at 6 dpi. Graphs show average percent of cells expressing single cytokines (IFN- γ , IL-4 and IL-10) and double cytokine-producing cells in the CD45.1⁺PD1^{hi}CD4⁺ T cell population (n=3, mean \pm SEM). *p <0.0, ***p <0.001.

To further test TFH cell intrinsic effects of STAT3, we utilized a mixed bone marrow (BM) chimera system where BM from CD45.2⁺ STAT3KO mice was mixed with CD45.1⁺ WT BoyJ BM and transferred into lethally irradiated WT BoyJ mice. Mice where BM from CD45.2⁺ WT (STAT3^{fl/fl}) mice was mixed with CD45.1⁺ WT BoyJ BM and similarly transferred into lethally irradiated CD45.1⁺ WT BoyJ mice were used as the control for the STAT3KO chimeras. The mice were allowed to repopulate their lymphoid systems for at least 90 days, and then were immunized with SRBC. Both WT and STAT3KO donor BM CD4 T cells repopulated the spleen normally in the absence of STAT3 (Fig. 14B). However, in contrast to our results with whole conditional mutant animals, STAT3-deficient CD4⁺ T cells in chimeric mice formed TFH cells at a markedly lower rate than control cells in the PP, whereas splenic TFH cells were less affected (Fig. 14C). These results suggest that in a competitive environment with wild-type TFH cells, STAT3-deficient TFH cells can develop to a significant degree, but in a chronic, ongoing response as in the PP, do not persist as well as wild-type TFH cells. The proportion of GC B cells was not affected by the presence of STAT3-deficient TFH cells, and likely the wild-type TFH cells compensated for any defect of the STAT3-deficient TFH cells (Fig. 14D). GC B cells showed a non-significant trend towards increased IgG1, which would fit with a model where increased IL-4 made by STAT3-deficient TFH cells was diluted out by wild-type TFH cells (Fig. 14E). In the chimeric PP, we examined cytokine production and consistent with our results in non-chimeric mice, we observed increased IFN- γ and IL-4, and decreased IL-21 in STAT3-deficient TFH cells (Fig. 15E-G). Thus, the altered

cytokine expression in STAT3-deficient TFH cells is cell intrinsic. In the chimeric spleen, we further examined the expression of Bcl6 and GATA3, and found these two key transcription factors were significantly increased in STAT3-deficient TFH cells in both PP and SP (Fig. 14F-G). Thus, deregulated Bcl6 and GATA3 expression in STAT3-deficient TFH cells is an intrinsic effect of loss of STAT3 in the TFH cells, and is not a function of an abnormal immune environment present in STAT3KO mice.

To better understand how STAT3 was required for TFH cell development, we wondered if STAT3-deficient CD4⁺ T cells proliferated poorly or underwent greater apoptosis, thus leading to non-competitive TFH cell responses. To test this, we used T cells from WT and STAT3KO OTII TCR mice, where the cells were labeled with a cell tracking dye prior to transfer. Six days after OVA-Alum immunization, we found that the STAT3-deficient T cells demonstrated slightly greater proliferation than the control cells (Fig. 16A-B). We additionally analyzed the rate at which STAT3-deficient TFH cells underwent apoptosis, using two different methods. We tested the ex vivo level of TFH cells initiating apoptosis by staining TFH cells for AnnexinV, both in spleen after SRBC immunization and in PP. We also tested whether the STAT3-deficient TFH cells might undergo higher apoptosis if they were stimulated through the TCR. TFH cells were isolated by FACS from spleens of SRBC-immunized mice, stimulated overnight with anti-CD3 antibody, and then measured for levels of active caspase 3. Contrary to expectations from the lack of in vivo competitiveness in chimeric mice, the

STAT3-deficient TFH cells had fewer or equal levels of apoptotic cells (Fig. 16C-E).

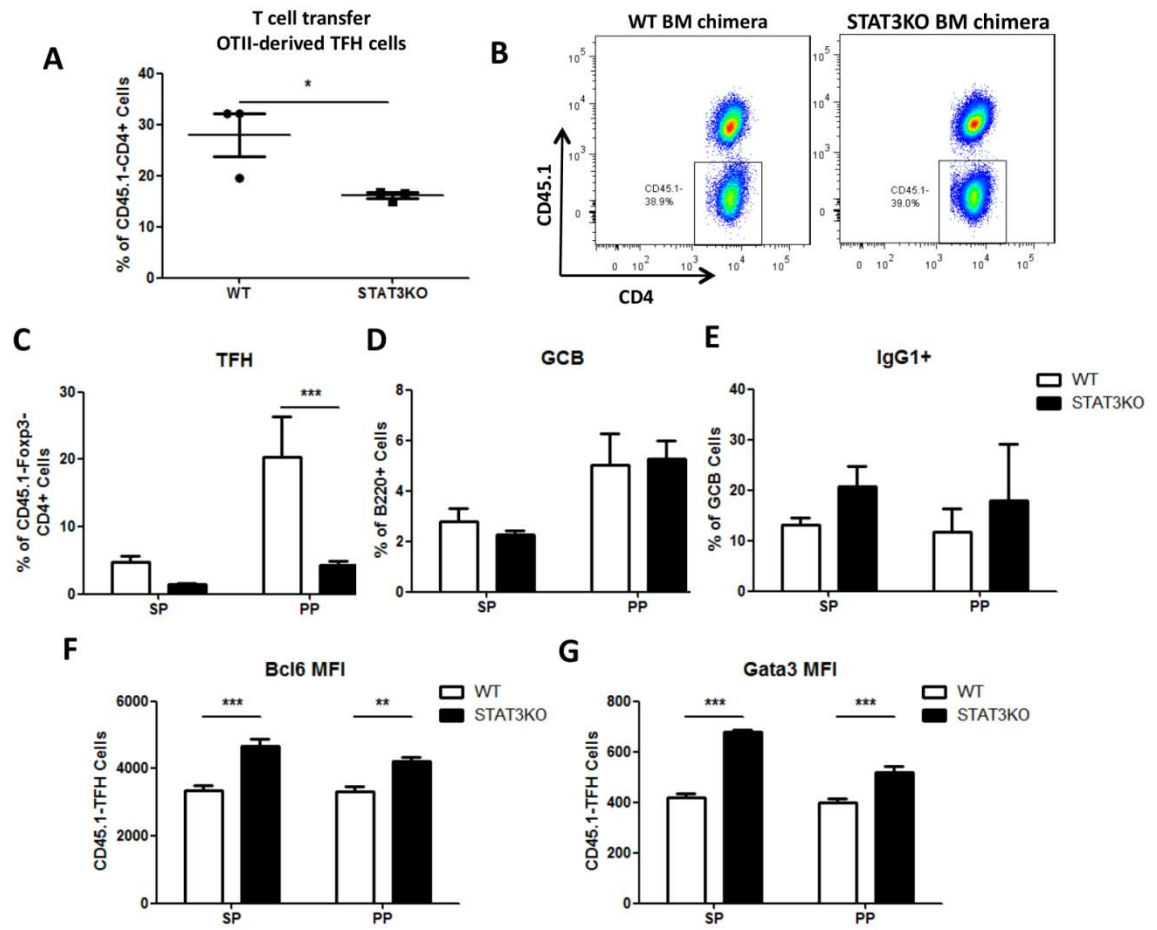


Figure 14. Intrinsic defect of STAT3-deficient TFH cell development.

(A) WT OTII TCR transgenic and STAT3KO OTII TCR transgenic CD45.2⁺CD4⁺ T cells were transferred to separate cohorts of CD45.1⁺ BoyJ mice, and recipient BoyJ mice were immunized with OVA-Alum. Graph shows average percent TFH cells within the transferred T cell population, analyzed at 6 dpi. (B-G) CD45.1⁺ BoyJ mice were lethally irradiated and divided into two groups. The WT group was given a mixture of bone marrow (BM) from BoyJ mice and CD45.2⁺ STAT3^{fl/fl} (WT) mice. The STAT3KO group was given a mixture of bone marrow (BM) from BoyJ mice and STAT3KO mice. After 90 days, the mice were immunized with SRBC. At 7 dpi, the mice were analyzed for percentages of transferred BM-derived CD45.1⁺ CD4 T cells in spleen (B), and percent of TFH cells (C) and GC B (D) in spleen (SP) and PP. Graphs show average percentage of CD45.1⁺ (CD45.2⁺) cells in the WT and STAT3KO groups. TFH cells were analyzed by flow cytometry as in Fig. 7 and 10. (E) IgG1⁺ GC B cell percentages in spleen and PP (n=4, mean \pm SEM). ***p <0.05 (t-test). (D-E) MFI of CD45.1⁺ TFH cells in spleen (SP) and PP for Bcl6 (F) and GATA3 (G). (n=4, mean \pm SEM). *p <0.05, **p <0.01, ***p <0.001 (t-test). Data are representative of two independent experiments with similar results.

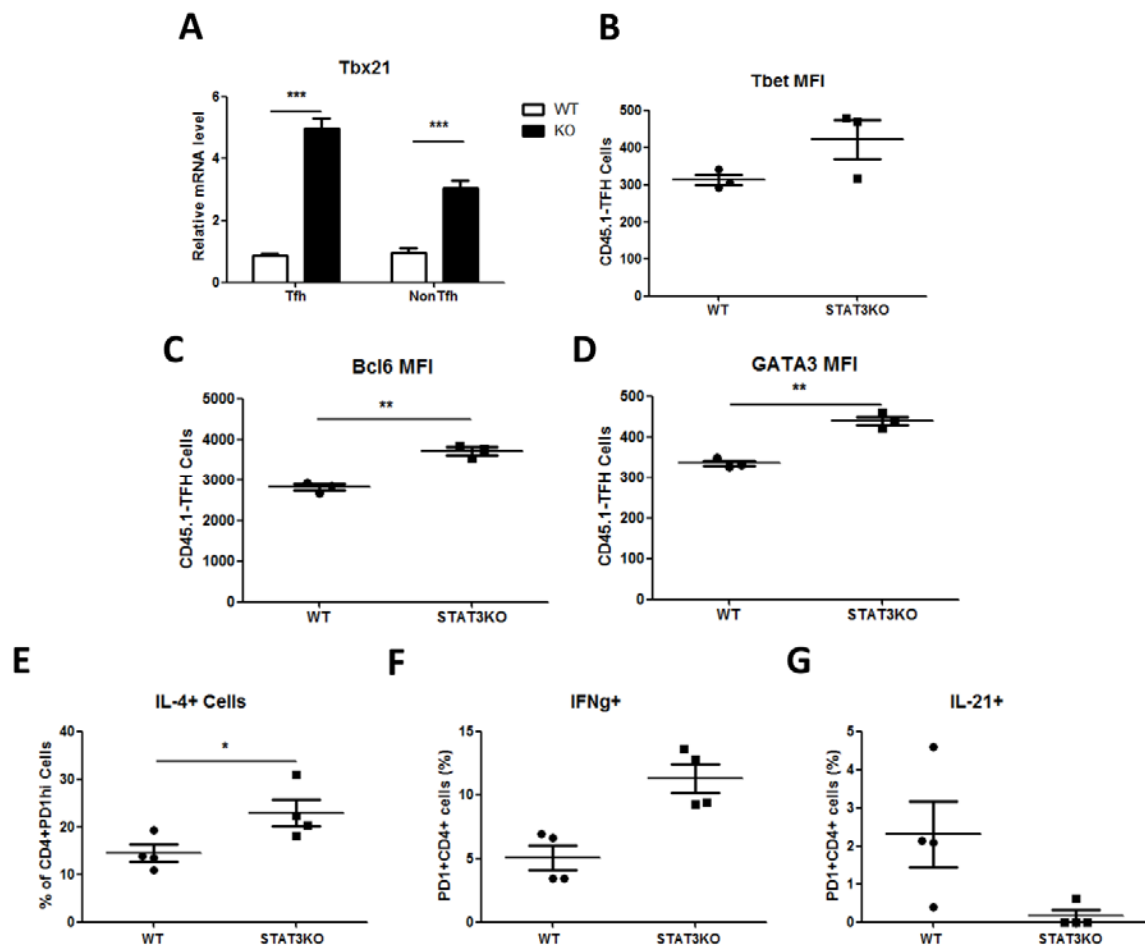


Figure 15. TFH cell intrinsic regulation of transcription factors and cytokines by STAT3.

(A) TFH cells in the PPs of WT and STAT3KO mice were sorted by FACS and RNA isolated post-sort. Tbx21 (Tbet) mRNA levels were assayed by QPCR. (B-D) WT and STAT3KO OTII TCR transgenic CD45.2⁺CD4⁺ T cells were transferred to CD45.1⁺ BoyJ mice, and the recipient BoyJ mice were immunized with OVA-Alum. TFH cells were analyzed at 6 days after immunization. Graphs show Tbet, Bcl6 and GATA3 MFIs of CD45.1-TFH cells, where each symbol represents one mouse (n=3, mean \pm SEM). (E-G) Cytokines in TFH cell populations in the PPs of bone marrow chimeras were measured by intracellular cytokine staining (ICS) following PMA plus ionomycin staining in vitro.

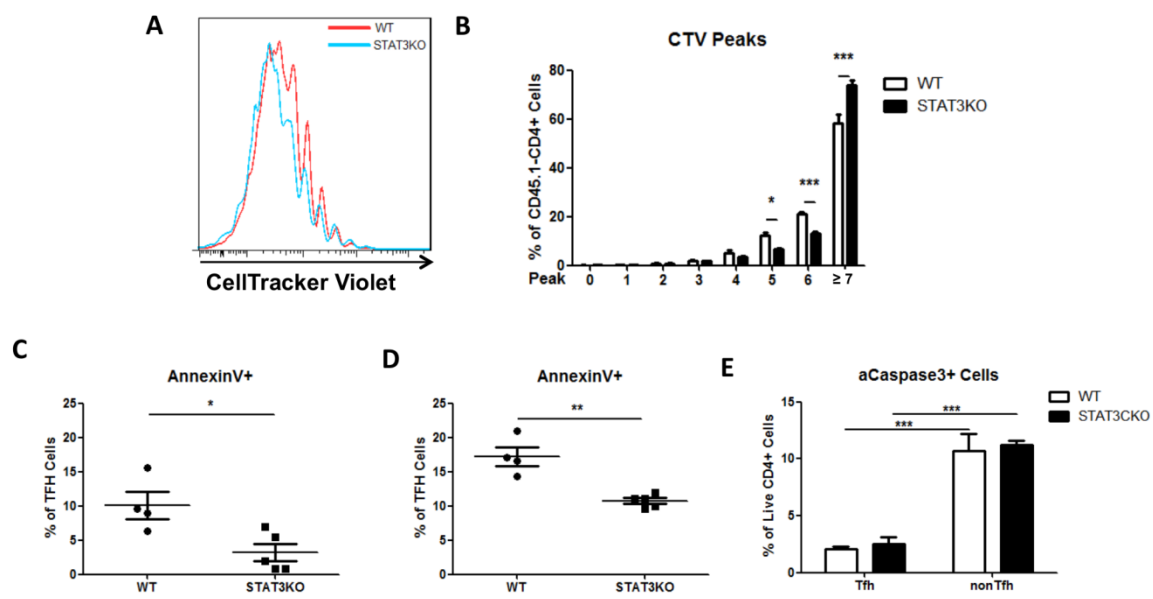


Figure 16. Increased proliferation and decreased cell death of STAT3-deficient TFH cells.

(A-B) WT and STAT3KO OTII TCR transgenic CD45.2⁺ CD4⁺ T cells were transferred to CD45.1⁺ BoyJ mice and immunized with OVA-Alum as in Fig. 14. (A) Histogram plot of Cell Tracker Violet (CTV)-labeled WT and STAT3KO OTII CD4⁺ T cells at 6 dpi with OVA-Alum. (B) Graphs of average MFI of CTV-labeled WT and STAT3KO OTII CD4⁺ T cells. Each symbol represents one mouse (n=3, mean \pm SEM). **p <0.01 (t-test). (C) Percentage of Annexin V⁺ TFH cells assayed ex vivo from spleen 7 dpi with SRBC in WT and STAT3KO mice. Each symbol represents one mouse (n=4-5, mean \pm SEM). *p <0.05 (t-test). (D) Percentage of Annexin V⁺ TFH cells assayed ex vivo from PP (n=4-5, mean \pm SEM). **p <0.01 (t-test). (E) 7 dpi with SRBC, average percentage of activated Caspase3⁺ (aCaspase3⁺) cells in FACS-isolated spleen TFH cells and non-TFH cell populations, stimulated with anti-CD3 antibody overnight in vitro . (n=3, mean \pm SEM). ***p <0.001 (ANOVA).

2.3.4: Control of Bcl6 repressive activity by STAT3.

To better dissect why STAT3-deficient TFH cells expressed more IL-4, we used an OVA antigen plus APC in vitro culture system where OTII TCR control and STAT3-deficient OTII TCR CD4⁺ T cells were activated under Th2 conditions, and IFN- γ and IL-4 expression were monitored by ICS after 3 days (Fig. 17A). Similar to what we observed for STAT3-deficient TFH cells, STAT3-deficient CD4⁺ T cells cultured under Th2 conditions produced about twice as much IL-4, and like the STAT3-deficient TFH cells, double cytokine-expressing cells were also increased (Fig. 17B). However, the increased IL-4 production by STAT3KO T cells was transient, as STAT3KO T cells cultured longer under Th2 conditions produced significantly less IL-4 than the control Th2 cells (Fig. 17C). Bcl6 is known to suppress IL-4 expression in CD4⁺ T cells in part by suppressing GATA3 transcriptional activity [94, 95], so we wondered why the increased Bcl6 expression in STAT3-deficient TFH cells failed to control IL-4. We therefore tested whether forced expression of Bcl6 by retrovirus (RV) could suppress IL-4 in STAT3-deficient CD4⁺ T cells cultured under Th2 conditions. For this experiment, CD4⁺ T cells from control and STAT3KO mice were activated under Th2 conditions using anti-CD3 and anti-CD28 antibodies plus APC. Under these conditions, about 50% of control T cells expressed IL-4 and a higher level, about 70% of STAT3-deficient T cells expressed IL-4 (Fig. 17D). Whereas Bcl6 RV was able to suppress over 60% of the IL-4 in control T cells, it suppressed less than 30% of the IL-4 in STAT3-deficient T cells (Fig. 17D, G). This strong loss of Bcl6 repressive activity was unique to IL-4, as Bcl6 repressed IL-10 slightly better

in STAT3-deficient T cells (Fig. 17E, G) and Bcl6 showed only a slight defect in repressing IL-13 (Fig. 17F, G). These data show that Bcl6 is defective in repressing IL-4 expression in the absence of STAT3, and this can explain the abnormal expression of IL-4 by STAT3-deficient TFH cells.

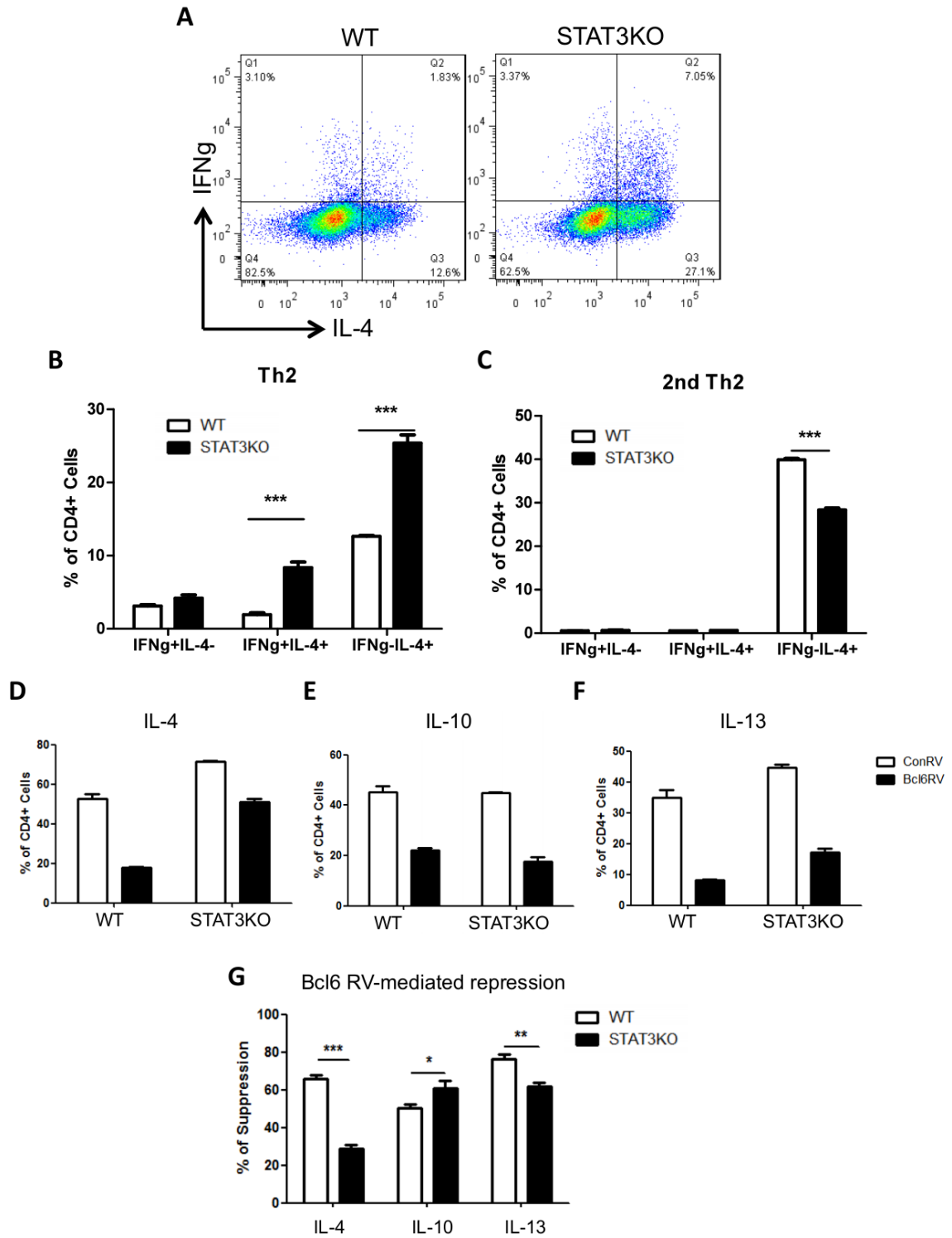


Figure 17. Defective Suppression of IL-4 by Bcl6 in STAT3-deficient T cells.

(A-B) WT and STAT3KO OTII TCR transgenic CD4⁺ T cells were cultured under Th2 conditions with APC and OVA peptide in vitro for 3 days. Cells were then stimulated with PMA plus ionomycin and analyzed for IFN- γ and IL-4 by ICS. (A) Sample staining of WT versus STAT3KO T cells after one round of Th2 culture. (B) Average percentages of IFN- γ ⁺IL-4⁻, IFN- γ ⁺IL-4⁺ and IFN- γ ⁻IL-4⁺ cell in CD4⁺ T cells after one round culture under Th2 conditions for 3 days (n=3, mean \pm SEM). (C) Average percentages of IFN- γ ⁺IL-4⁻, IFN- γ ⁺IL-4⁺ and IFN- γ ⁻IL-4⁺ cell in CD4⁺ T cells after an additional 5 days of Th2 culture ("2nd Th2", n=3, mean \pm SEM). (D-G) WT and STAT3KO CD4⁺ T cells under Th2 condition and infected with Bcl6-expressing and control RVs. Average percentages of T cells expressing (D) IL-4, (E) IL-10, (F) IL-13 assayed by ICS, after PMA plus ionomycin stimulation. (G) Analysis of data in (D-F) showing average percent suppression by Bcl6 RV in WT and STAT3KO CD4⁺ T cells. Percent suppression = ((% cytokine⁺ cells with control RV minus % cytokine⁺ cells with Bcl6 RV) divided by % cytokine⁺ cells with control RV) x 100. Data are representative of two independent experiments with similar results. *p <0.05, **p <0.01, ***p <0.001 (ANOVA).

2.4: Discussion.

TFH cells control germinal center reaction and the production of high-affinity antibodies to antigen. The pathways for how TFH cells develop and regulate B cell responses are a major part of the adaptive immune response. When we initiated this study, we were interested in elucidating how critical STAT3 was for TFH cell differentiation and if other pathways besides STAT3 could induce Bcl6 expression in TFH cells. Recent studies have revealed that TFH cells can develop at a low level in the absence of STAT3 [56], and that STAT4 and STAT1 can participate in inducing Bcl6 during TFH cell differentiation [76, 78, 79]. The work of Ray et al [56] established that STAT3 is critical factor for not only activating Bcl6 in TFH cells but also for repressing a Type I IFN pathway that induces a Th1-like effector program in TFH cells. However, since this study was done solely in the context of acute LCMV infection where a great deal of Type I IFN is produced, whether this pathway represented the dominant STAT3-mediated control pathway in TFH cells, or only was operative in the case of virus infection, was unclear. Thus, we analyzed T cell specific STAT3 conditional KO mice (CD4-cre STAT3^{fl/fl}; STAT3KO) for other types of TFH cell responses. In the ongoing chronic TFH cell/GC B cell response of the PP, we found that loss of STAT3 caused slight decrease of the TFH cells within the PP, while with no effects on the number of GC B cells. Furthermore, there was a shift towards the expression of both Th1 (IFN- γ) and Th2 (IL-4) cytokines in STAT3-deficient PP TFH cells. We observed that Bcl6 expression was notably higher in STAT3-deficient PP TFH cells. These data indicate that STAT3 function in TFH cells can

vary depending on the type of immune response, and that STAT3 appears to insulate TFH cells from differentiating into different effector pathways depending on the cellular micro-environment. Most dramatic, though was our finding that PP GC B cell responses were present at normal levels in the STAT3KO mice, with increased Bcl6 expression by TFH cells. The function of STAT3 in regulating Bcl6 expression in TFH cells is therefore heavily influenced by the local immune environment. In a virus infection with high levels of Type I IFN, STAT3 is required for up-regulating Bcl6 in TFH cells, but in the immune response against commensal organisms in the PP, STAT3 actually restrains the up-regulation of Bcl6 in TFH cells. In the anti-virus response, Type I IFN suppresses Bcl6 expression, and it was proposed that Type I IFN leads to IL-2 gene transcription and downstream STAT5 activation, where STAT5 then directly binds to and represses Bcl6 [56]. In contrast, for the gut-PP immune environment and for antigens where Type I IFN is not produced, such as OVA-Alum, STAT5 is likely not activated in TFH cells. We propose that the lack of STAT3 without STAT5 activation allows other STATs, such as STAT1 and STAT4, to bind to the Bcl6 promoter and more strongly activate transcription than when STAT5 is active.

A related question is why IL4 is up-regulated in STAT3-deficient TFH cells. We observed this effect in different types of TFH cell responses (PP, SRBC immunization, OVA-Alum immunization and in vitro activation under Th2 conditions). Thus it seems to be a general effect in our hands. Notably, IFN- γ was increased in STAT3-deficient PP TFH cells and the in vitro cultured TFH cells, but not in STAT3-deficient TFH cells induced by SRBC or OVA/Alum. Thus,

the immune environment is key for the exact type of cytokine response that STAT3 regulates in TFH cells. Our data with Bcl6 RV show that Bcl6 is unable to suppress IL-4 in the absence of STAT3, but the mechanism for this effect is not clear. One possibility is the increased GATA3 we observe in CD4⁺ T cells in the absence of STAT3. GATA3 may put the IL-4 gene chromatin in a hyper-activated state that is resistant to repression by Bcl6. A related possibility is that there is differential regulation of IL-4 by STAT factors in the absence of STAT3. This model is analogous to the model for regulation of Bcl6 transcription in the absence of STAT3 described above so that IL-4 gene expression is controlled by loss of STAT3 very differently in the virus infection system versus immune responses where Type I IFN is not produced, such as in the PP. In the absence of Type I IFN and STAT1 activity, other STAT factors may bind to IL-4 or GATA3 regulatory regions and induce high-level expression of these genes. Yet another possibility is that STAT3 regulates the transcription of a co-factor required for repression of IL-4 by Bcl6. The exact mechanism for how Bcl6 represses IL-4 expression in CD4⁺ T cells is not known, though the current model is that Bcl6 represses GATA3 transcriptional activity, and thus indirectly suppresses IL-4 gene expression [95, 96].

Our data showing increased IL-4 by T cells in the absence of STAT3 contrasts with published work showing that STAT3 is required for optimal full Th2 cell differentiation [54]. A possible explanation for this discrepancy is that TFH cells regulate IL-4 gene expression by different regulatory elements than the regulatory elements that Th2 cells use for IL-4 expression [97, 98], and overall it

is clear that TFH cells are a separate lineage from Th2 cells. At the same time, we observed increased IL-4 expression by STAT3-deficient T cells cultured under Th2 conditions (Fig. 17B). Critically, in the in vitro Th2 cultures, we analyzed IL-4 and other cytokines in these STAT3-deficient Th2 cells at an early time point: 3 days after initial activation. When we examined STAT3-deficient Th2 cells cultured for longer periods and after re-stimulation (Fig. 17C), the increased IL-4 expression was lost. Indeed, data in the previous report also showed that IL-4 production within the first 24-48 hours was not deficient in the absence of STAT3 [99]. Thus, we can reconcile these findings then by proposing that STAT3 indeed suppresses expression of IL-4 shortly after activation, but that STAT3 is actually important for long-term Th2 cell stability and/or survival.

Curiously, we found that STAT3-deficient TFH cells survive about the same or slightly better than wild-type TFH cells (Fig. 16C-E), which contradicts the standard view that STAT3 is a pro-transformation and pro-survival transcription factor [77, 81, 100]. One explanation is that the increased survival of STAT3-deficient TFH cells is due to increased Bcl6 expression, since we have previously shown a survival effect of Bcl6 in TFH cells [93]. Whether this effect is unique to TFH cells and holds up to further scrutiny will require investigation.

In summary, we have revealed a much more complete picture of how STAT3 regulates TFH cell development and function. STAT3 function in TFH cells appears to be strongly dependent on the immune environment, and STAT3 plays very different regulatory roles in TFH cells that develop during virus infection than in TFH cells that develop in response to commensal organisms in

the gut, as well to model antigens that do not provoke a strong type I IFN response.

Chapter 3 - Follicular regulatory T cells repress cytokine production by follicular helper T cells and optimize IgG responses in mice

3.1: Introduction.

During an immune response, CD4⁺ T cells can differentiate into several unique effector lineages that promote different immune responses via the secretion of distinct types of cytokines. TFH cells are a recently characterized CD4 lineage whose major function is to help B cells form GCs and produce high-affinity antibodies [42, 66]. Both in mouse and human, TFH cells control the initiation as well as the outcome of the GC B-cell response [69, 101-103]. While TFH cells are critical for the proper production of antibodies, the over-production of TFH cells can lead to autoimmunity, since TFH cells can help B cells to produce self-reactive antibodies [69-71]. Thus, the proper regulation of TFH- and GC B-cell responses is essential both for normal immune function and for preventing autoimmune disease.

A subpopulation of Treg cells in the GCs was discovered that appear to act as suppressors of TFH and GC B cells [60-62]. These follicular regulatory T (TFR) cells display a mixed phenotype of both TFH cells and Tregs. Like TFH cells, TFR cells express CXCR5, ICOS, and PD-1, and depend on Bcl6 for differentiation and localization to the B cell follicle. Although TFR and TFH cells are phenotypically similar, TFR cells originate from natural Tregs, whereas TFH cells originate from naive CD4⁺ T cells [61]. In vitro and in vivo studies have shown that TFR cells can suppress TFH- and GC B-cell proliferation and can

regulate GC B cell differentiation as well [60-64]. A study using a NFAT2 conditional knockout mouse model showed that decreased development of TFR cells correlated with increased TFH- and GC B-cell responses, and these mice also developed anti-dsDNA auto-antibodies and lupus-like disease [104].

However how TFR cells affect antibody production is still unresolved. Several studies have shown that TFR cells repress antibody production [60-64]. Furthermore, Linterman et al demonstrated that TFR cells control the outgrowth of non-antigen-specific GC B cells and helped maintain high titers of high-affinity antigen-specific antibodies [61]. However, the precise mechanisms by which TFR cells control antibody responses are not known.

Despite a large number of studies analyzing TFR cells in GC responses, a mouse model where TFR cells are specifically deleted and that allows for systematic study has been lacking. Experimental systems with adoptive transfer of TFR cells into T cell deficient mice can have non-physiological effects [60, 61, 64, 105]. Bone marrow chimeras have been used to analyze loss of TFR cells [61], but the number of animals that can be tested is limited and this approach relies on the production of TFH cells from Foxp3-deficient T cells. Total Treg deletion [60-62, 104, 106, 107] does not specifically target TFR cells, and causes a much broader effect on the immune response that can mask the actual functions of TFR cells.

Here, we have undertaken a further characterization of TFR cells with a novel mouse model. We find that TFR cells have low expression of CD25 and high expression of the inhibitory molecule T-cell immunoreceptor with Ig and ITIM

domains (TIGIT). We used $Bcl6^{fl/fl}Foxp3^{cre}$ (Bcl6FC) mice to completely delete TFR cells and leave remaining Tregs intact. Although loss of TFR cells has no gross effect on size of TFH- or GC B-cell populations after immunization, we find that TFR cells promote the generation of normal levels of IgG and repress IgA responses. Further, TFR cells repress the production of anti-dsDNA IgA antibodies in a lupus model. TFH cells from Bcl6FC mice consistently produce higher levels of IFN- γ , IL-10 and IL-21, which are well-known cytokines that promote B-cell responses. We hypothesize that the increased cytokine production by TFH cells in the absence of TFR cells may lead to the altered antibody responses. Overall, our findings uncover both positive and negative regulatory roles for TFR cells in antibody responses, in both vaccine and autoimmune settings.

3.2: Materials and Methods.

Mice. $Bcl6^{fl/fl}$ mice were previously described [108]. Foxp3-YFP-cre mice [109] were obtained from Jackson labs. Genotyping for the floxed $Bcl6$ allele was performed as described [108]. Genotyping for Foxp3-YFP-cre allele was according to PCR protocols from Jackson labs. $Bcl6^{fl/fl}$ mice were crossed to Foxp3-YFP-cre mice. Control mice for Bcl6FC conditional KO mice were Foxp3-cre $Bcl6^{+/+}$ (wild-type [WT]) mice. Mice were bred under specific pathogen-free conditions at the laboratory animal facility at IUSM and were handled according to protocols approved by the IUSM Animal Care and use Committee.

Immunization. For SRBC immunization, mice were i.p. injected with 1×10^9 SRBC (Rockland Immunochemicals) and were sacrificed at the indicated day. For KLH (Sigma-Aldrich) immunization, 100 ug KLH was mixed with Imject Alum (Pierce) and the mixture was injected i.p.. For gp120 DNA prime-protein boost vaccine immunization as previous reported [110, 111], gp120 DNA vaccine construct in pJW4303 vector and gp120 protein produced from Chinese Hamster Ovary (CHO) cells were used. Mice were primed by i.m. injections of 100 ug of gp120 construct every two weeks for three times. Four weeks after the third DNA immunization, mice were boosted with 10 ug gp120 protein plus Alum mixture twice at two week interval. Mice were sacrificed one week after the second gp120 protein boost. For both DNA and protein immunizations, a total of 100 ul was injected, 50 ul per hind leg.

Pristane administration. WT and Bcl6FC mice were given a single i.p. injection of 0.5 ml Pristane (Sigma-Aldrich) at 12 weeks of age. After 4 months, mice were sacrificed. The presence of anti-dsDNA antibodies in the serum was tested by ELISA, and spleens were taken out for further flow cytometry analysis.

Flow cytometry reagents. Anti-CXCR5 (2G8), GL7 (GL7), and anti-IL-4 (11B11) antibodies were from BD Biosciences. Fixable viability dye, anti-CD38 (90), anti-TIGIT (GIGD7), anti-IL-21 (mhalx21), and anti-Foxp3 (FJK-16s) antibodies were from eBioscience. Anti-CD4 (GK1.5), anti-B220 (RA3-6B2), anti-PD1 (29F.1A12), anti-CD25 (PC61), anti-IL-10 (JES5-16E3), Annexin V and anti-IFN- γ (XMG1.2) were from Biolegend.

Cell staining for flow cytometry. Cell suspension from spleen were first incubated with anti-mouse CD16/CD32 (Bio X Cell) for 5 min at room temperature, followed by surface staining of indicated markers and viability staining. For intracellular staining, after surface staining, cell were fixed and stained with antibodies against intracellular proteins by following Foxp3 fixation kit (eBioscience) protocol. Cell events were collected on LSRII flow cytometer (BD Biosciences) and analyzed by Flowjo software.

Intracellular cytokine staining. 2×10^6 cells were stimulated with PMA (75 ng/ml) plus ionomycin (1 ug/ml) for 5 hours in DMEM with 10% FBS, then fixed and stained for indicated cytokines as described [112]. GolgiStop and GolgiPlug (BD Biosciences) were added with the stimulation to inhibit cytokine release.

Preparation of SRBC membrane antigens for ELISA. All procedures were carried out at 4°C. After each centrifugation, the supernatant were removed by vacuum. SRBC (Rockland Immunochemicals) were centrifuged at 13,000g for 10 min. The pellet was re-suspended in lysis buffer (0.05 M Tris-HCL with 0.1 mM EDTA, pH 7.6), vortexed thoroughly, and then centrifuged again. This process was repeated 7 to 10 times to remove all visible traces of hemoglobin. Before last centrifugation, the pellet was re-suspended and passed through a 40 µm cell strainer. After the final wash, the pellet from 5 ml SRBC was re-suspended in 500 ul 0.1% SDS and stored at -20 °C.

Anti-SRBC, anti-dsDNA and anti-gp120 antibody titers analysis. Anti-SRBC IgG/IgM, and anti-dsDNA IgA/IgG/IgM titers were determined by ELISA,

as previously reported [108]. Briefly, 96-well Nunc-Immuno plates (Sigma-Aldrich) were coated with SRBC membrane antigens or salmon sperm DNA passed through 45 μ m filter (Invitrogen) overnight at 4°C. Wells were blocked with 1% BSA for 1.5 hours. Diluted serum samples were applied. ELISA plates were incubated for 2 hours at room temperature for anti-SRBC antibody detection, or incubated for 1 hour at 37°C for anti-dsDNA antibody detection. Peroxidase-conjugated anti-mouse IgA, anti-mouse IgG or anti-mouse IgM antibodies (Sigma-Aldrich) was used as secondary antibody. Anti-dsDNA ELISA plates were washed extensively after each incubation to minimize high background signals. Anti-gp120 IgG titers were measured by ELISA as previously described [110, 111]. Anti-gp120 antibody avidity was measured via the NaSCN displacement method, as previously reported [110, 111].

Statistical analysis. All data analysis was done in GraphPad Prism software. Unless otherwise stated, a Student t test or ANOVA with a Tukey post hoc analysis were used. Only significant differences ($p < 0.05$) are indicated in figures.

3.3: Results.

3.3.1: Characterization of TFH and TFR cell populations.

Initially, we analyzed wild-type mice, in order to assess the development of TFH and TFR cells after immunization. We used SRBC, which is known to induce a strong TFH and GC response. As shown in Fig. 18A, both the Foxp3- and Foxp3⁺ cell populations contain a fraction of cells expressing both CXCR5

and PD-1, with a gradient from intermediate expression (CXCR5^{low}PD-1^{low}) to high expression (CXCR5^{hi}PD-1^{hi}). TFH cells are commonly defined based on the expression of these two cell surface markers [65]. For this study, in Foxp3⁺CD4⁺ T cells, we define CXCR5^{hi}PD-1^{hi}, CXCR5^{neg}PD-1⁺ and CXCR5^{neg}PD-1^{neg} cells as TFH, PD-1⁺TH and PD-1⁻TH cells, respectively. We applied the same cell subpopulation gating and definition to the Treg compartment, which are TFR, PD-1⁺Treg and PD-1⁻Tregs. TFH and TFR cells expressed the highest levels of Bcl6, the master transcription factor for TFH- and TFR-cell differentiation, among conventional CD4 T (Tconv) cells and Tregs, respectively (Fig. 18B, Fig. 19). Since IL-2 and STAT5 signaling is known to inhibit TFH-cell differentiation [51, 113, 114] and CD25 is often used as a Treg marker, we next compared CD25 expression on different Tconv or Tregs subpopulations directly ex vivo (Fig. 18C, Fig. 19). The overall level of CD25 on Tregs was much higher than Tconv cells. However, within Foxp3⁺ cells, TFR cells had significantly lower level of CD25 than other subpopulations. This indicates that down regulation of CD25 may be part of TFR-cell differentiation program similar to TFH cells. Previous studies have shown that TFH cells highly express the inhibitory molecule TIGIT [115, 116] and TIGIT is important for Treg suppressive function on Th1 and Th17 cell [117]. Therefore we checked TIGIT expression on TFR cells. As shown in Fig. 18D, both TFH and TFR cells express the highest level of TIGIT in Tconv and Tregs, respectively, and further that the MFI of TIGIT on TFR cells is more than two-fold higher than TFH cells. TFR cells are enriched within the TIGIT-high Treg population, but TFR cells only comprise a fraction of the TIGIT-high Treg

population (Fig. 20). Taken together, TFR cells express high levels of Bcl6 and TIGIT, while a relatively low level of CD25. These data define novel differences between TFR and TFH cells and also between TFR cells and conventional Tregs.

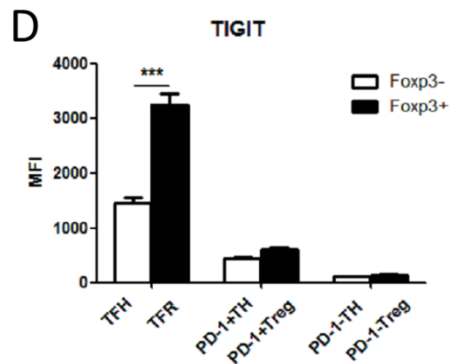
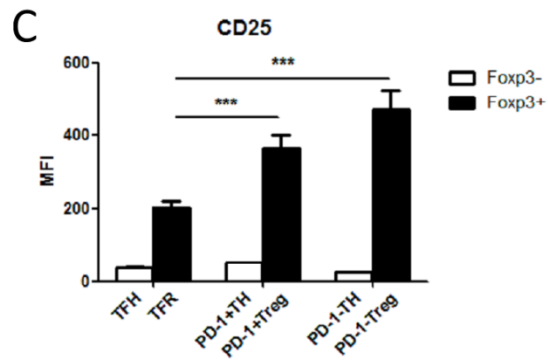
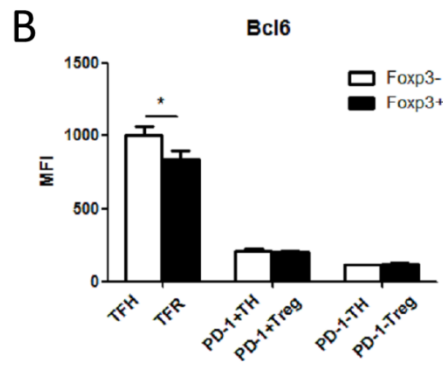
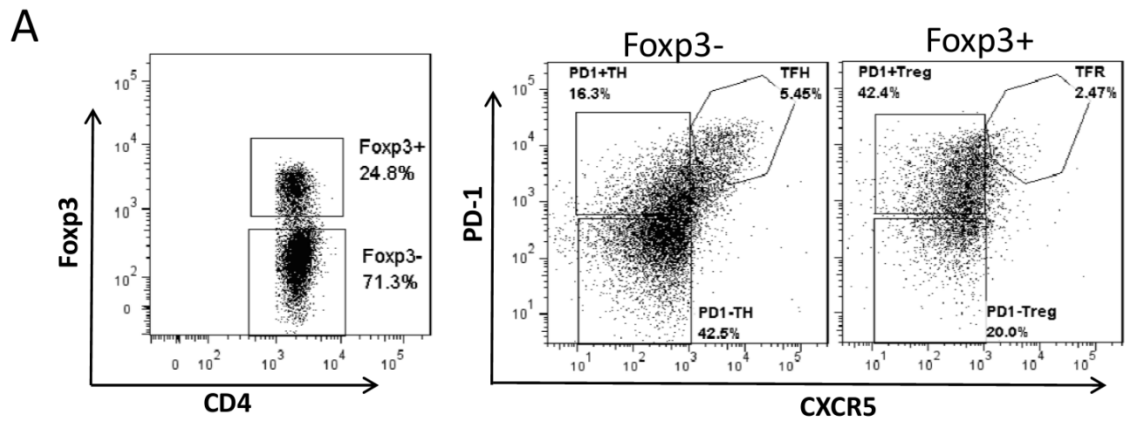


Figure 18. Bcl6, CD25 and TIGIT levels in various conventional and regulatory T cell subsets.

Splenic CD4⁺ T cells from WT mice immunized with SRBC 7 d before were studied by flow cytometry. (A) Foxp3⁺ and Foxp3⁻ CD4⁺ T cells are gated as in the flow plot (left). In Foxp3⁻ conventional CD4⁺ T (Tconv) cells, TFH cells, PD-1⁺TH cells and PD-1⁻ TH cells are defined as CXCR5^{hi}PD-1^{hi}, CXCR5^{neg}PD-1⁺ and CXCR5^{neg}PD-1^{neg}, respectively. In Foxp3⁺ regulatory CD4⁺ (Treg) cells, TFR, PD-1⁺Treg and PD-1⁻ Treg cells are gated on CXCR5^{hi}PD-1^{hi}, CXCR5⁻PD-1⁺ and CXCR5⁻PD-1⁻, respectively (right). MFI of Bcl6 (B), CD25 (C) and TIGIT (D) in TFH, PD-1⁺TH, PD-1⁻ TH, TFR, PD-1⁺Treg and PD-1⁻ Treg cells. Data shown as mean +/- SEM, n = 4-5. *p < 0.05, **p < 0.01, *** p < 0.001 (ANOVA). Data are representative of two independent experiments with similar results.

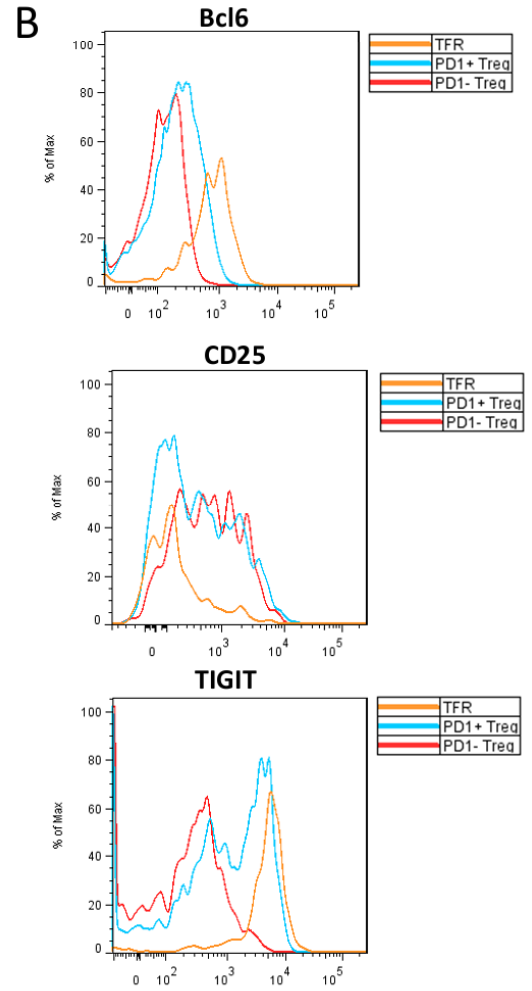
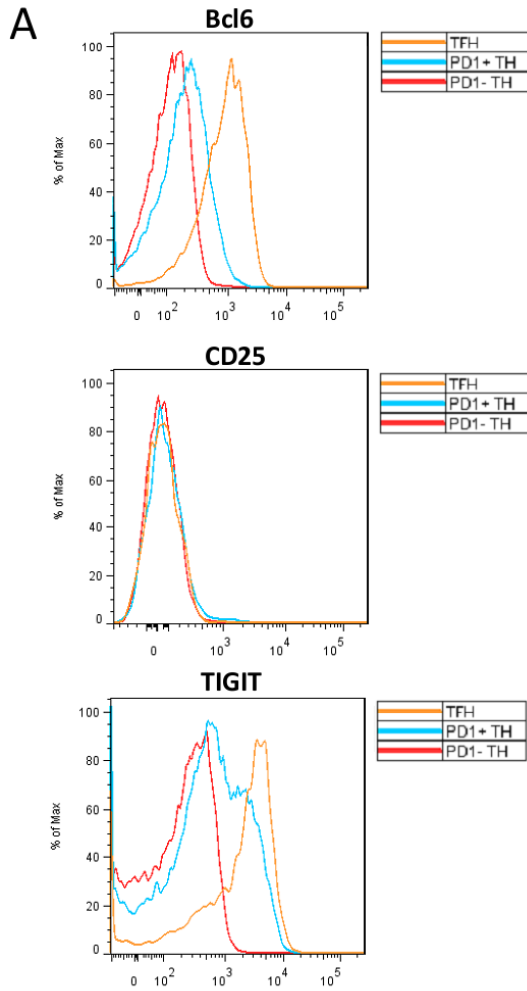


Figure 19. Histogram plots of Bcl6, CD25 and TIGIT staining in different of Tconv and Treg subpopulations.

Spleens were isolated from mice 7 days after SRBC immunization for flow cytometric analysis. Foxp3⁻ and Foxp3⁺ CD4⁺ T cell subsets are gated as in Fig. 18A. (A) Histogram plots show the expression of Bcl6, CD25 and TIGIT in TFH, PD-1⁺TH and PD-1⁻ TH cells. (B) Histogram plots show the expression of Bcl6, CD25 and TIGIT in TFR, PD-1⁺Treg and PD-1⁻ Treg cells. Plots are representative of two independent experiments with similar results.

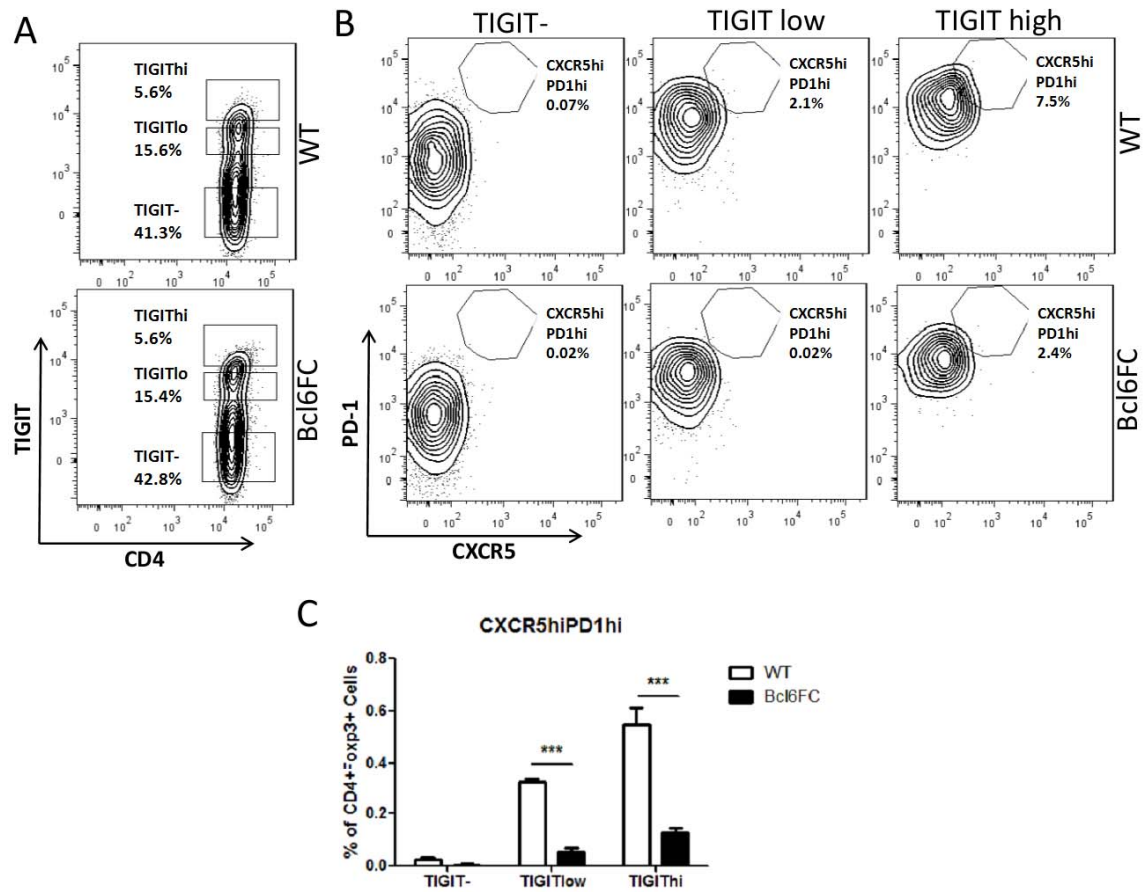


Figure 20. TFR cells are enriched within TIGIT-high Treg cells.

Splenic CD4⁺ T cells from control and Bcl6^{FC} mice immunized with SRBC 10 d before were studied by flow cytometry. (A) TIGIT negative, low and high Treg cells are gated as in the flow plots. (B) TFR cells are defined as Foxp3⁺CXCR5^{hi}PD-1^{hi}. The flow plots of TFR cells in TIGIT negative, low and high Treg cells are shown. (C) Within TIGIT negative, low and high Treg cell populations, TFR cell percentages are shown. Graphs show mean +/- SEM, n = 4. *** p < 0.001 (two-way ANOVA). Data are representative of two independent experiments with similar results.

3.3.2: Loss of TFR cells does not lead to an increased TFH cell population in Bcl6FC mice.

In order to assess the functional role of TFR cells more precisely, we created Bcl6FC mice, where the floxed Bcl6 allele is deleted specifically in Foxp3 expressing T cells. Similar to Bcl6^{fl/fl}CD4^{cre} mice [108], Bcl6FC mice are healthy, fertile, do not display abnormal inflammation and have grossly normal immune cell compartments [118]. This is in contrast to germline Bcl6^{-/-} mice, which are extremely prone to spontaneous inflammatory disease and early death [119]. Tregs from Bcl6^{-/-} mice are strongly skewed towards a pro-inflammatory Th2 phenotype, which is largely driven by the severe Th2 inflammation in these mice [96]. However, Tregs from unimmunized Bcl6FC mice do not show abnormal Th2 cytokine production [118], consistent with the lack of inflammatory disease in the mice. Prior to immunization, both control (Bcl6^{+/+}Foxp3^{cre}) mice and Bcl6FC mice have very low levels of TFH cells, less than 0.5% of total spleen, indicating that the Bcl6FC mice do not have increased basal levels of TFH cells. We then immunized control and Bcl6FC mice with SRBC and analyzed TFH and TFR cells in spleen at 10 dpi. As expected, the induction of CXCR5^{hi}PD-1^{hi}Foxp3⁺ TFR cells was strongly diminished in Bcl6FC mice (Fig. 21B). We wondered if the great loss of TFR cells is due to apoptosis in the absence of Bcl6, similar to our findings in Bcl6^{fl/fl}CD4^{cre} mice [108]. In those mice, the few TFH cells that develop are undergoing a high rate of apoptosis. We therefore examined survival of TFR cells in Bcl6FC mice, and found that indeed, TFR cells are undergoing a significantly higher rate of late apoptosis indicated as increased percentage of

Annexin V and viability dye double positive TFR cells (Fig. 22). However, in contrast to previous reports [60-62], we did not see an increase in the proportion or number of TFH cells in immunized Bcl6FC mice (Fig. 23B). This result is not unique to SRBC immunization, as Bcl6FC mice also had comparable TFH cell populations as control mice did at different time-points after immunization with SRBC, as well as in several other types of immune challenge with different antigens (data not shown). Additionally, we did not observe changes in the numbers of PD1- and CXCR5-intermediate Foxp3- T cells (early/pre-TFH cells) in Bcl6FC mice, and thus there was no altered regulation of TFH-cell differentiation at an earlier stage due to loss of TFR cells (Fig. 23D). Thus, TFR cells are specifically and strongly deleted in Bcl6FC conditional KO mice, however loss of TFR cells did not have any gross effect on the development of the TFH-cell population.

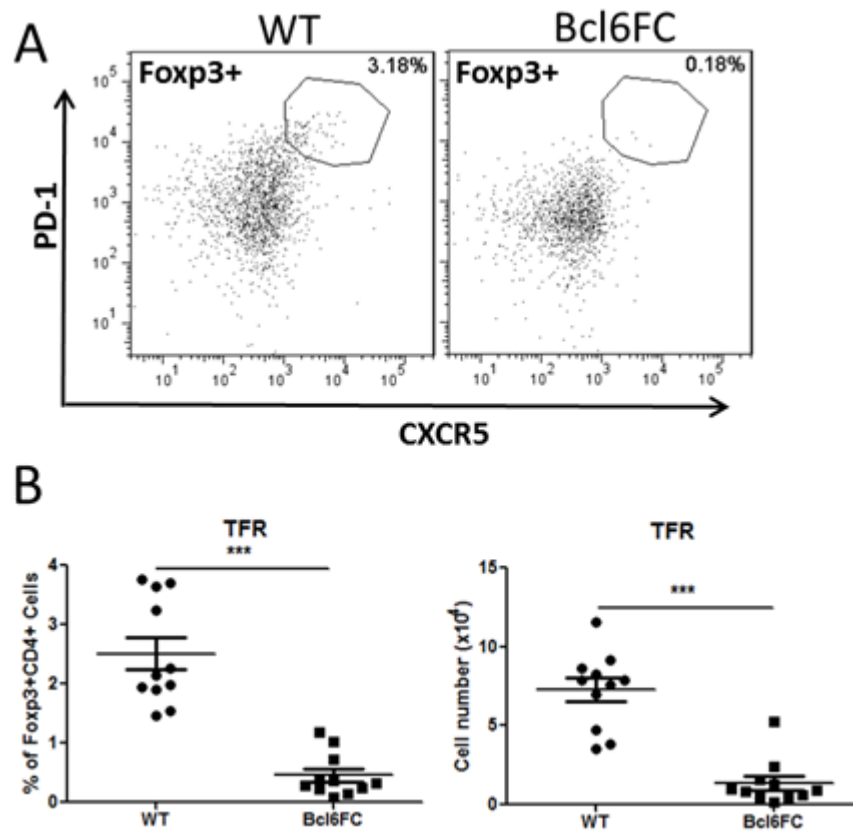


Figure 21. Loss of TFR cells in Bcl6FC mice following SRBC immunization.

Control and Bcl6FC KO mice were immunized with SRBC by i.p. injection. At 10 dpi, spleens were isolated for flow cytometric analysis. (A) TFR cells are defined as $\text{Foxp3}^+\text{CXCR5}^{\text{hi}}\text{PD-1}^{\text{hi}}$. (B) TFR cell percentage in $\text{CD4}^+\text{Foxp3}^+$ T cells and TFR cell number. Graphs show mean \pm SEM, $n = 6-7$. ** $p < 0.01$ (student t test). Data are combined from two independent experiments.

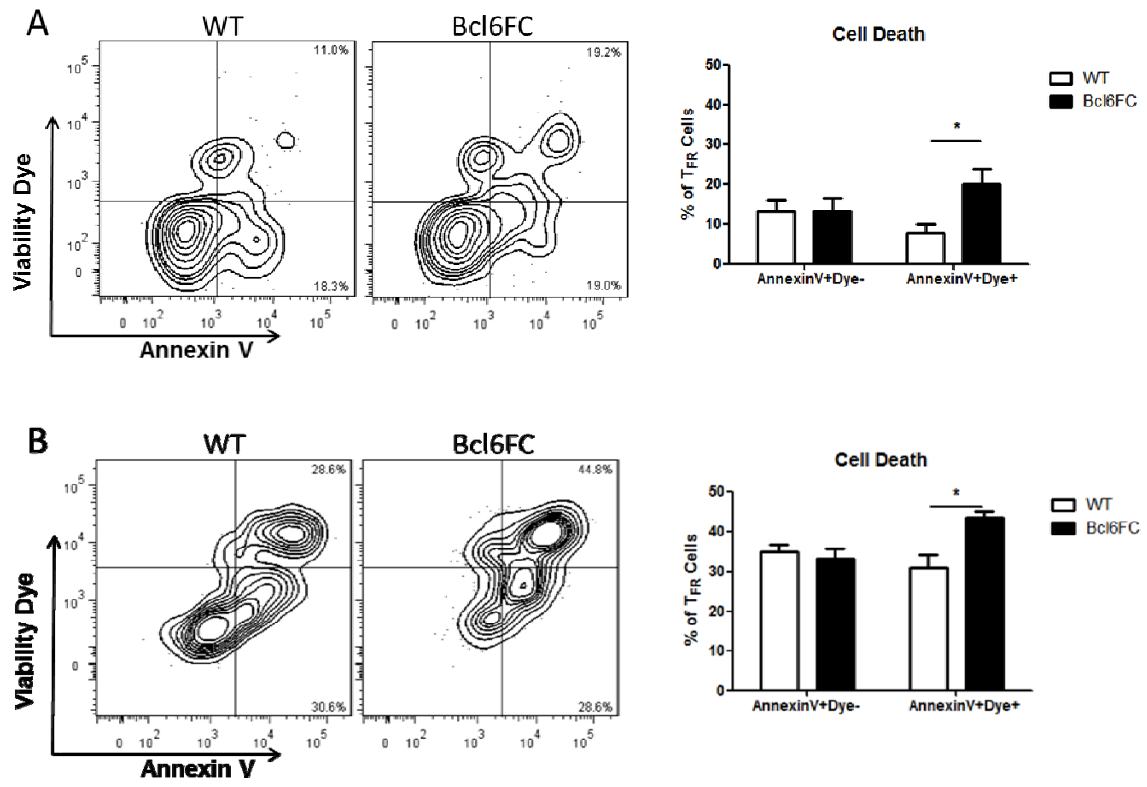


Figure 22. Bcl6 maintains the survival of TFR cells.

Survival of splenic TFR cells at 5 dpi in mice immunized with SRBC (A) and 25 dpi in mice immunized with NPKLH Alum (B) were studied. (A-B) Representative flow plots of viability dye and AnnexinV binding of TFR cells are shown on the left. Percentages of AnnexinV⁺Viability Dye⁻ and AnnexinV⁺Viability Dye⁺ cells in TFR cells are shown on the right. Graphs show mean +/- SEM, n = 4. *p < 0.05 (student t test). Data are representative of two independent experiments with similar results.

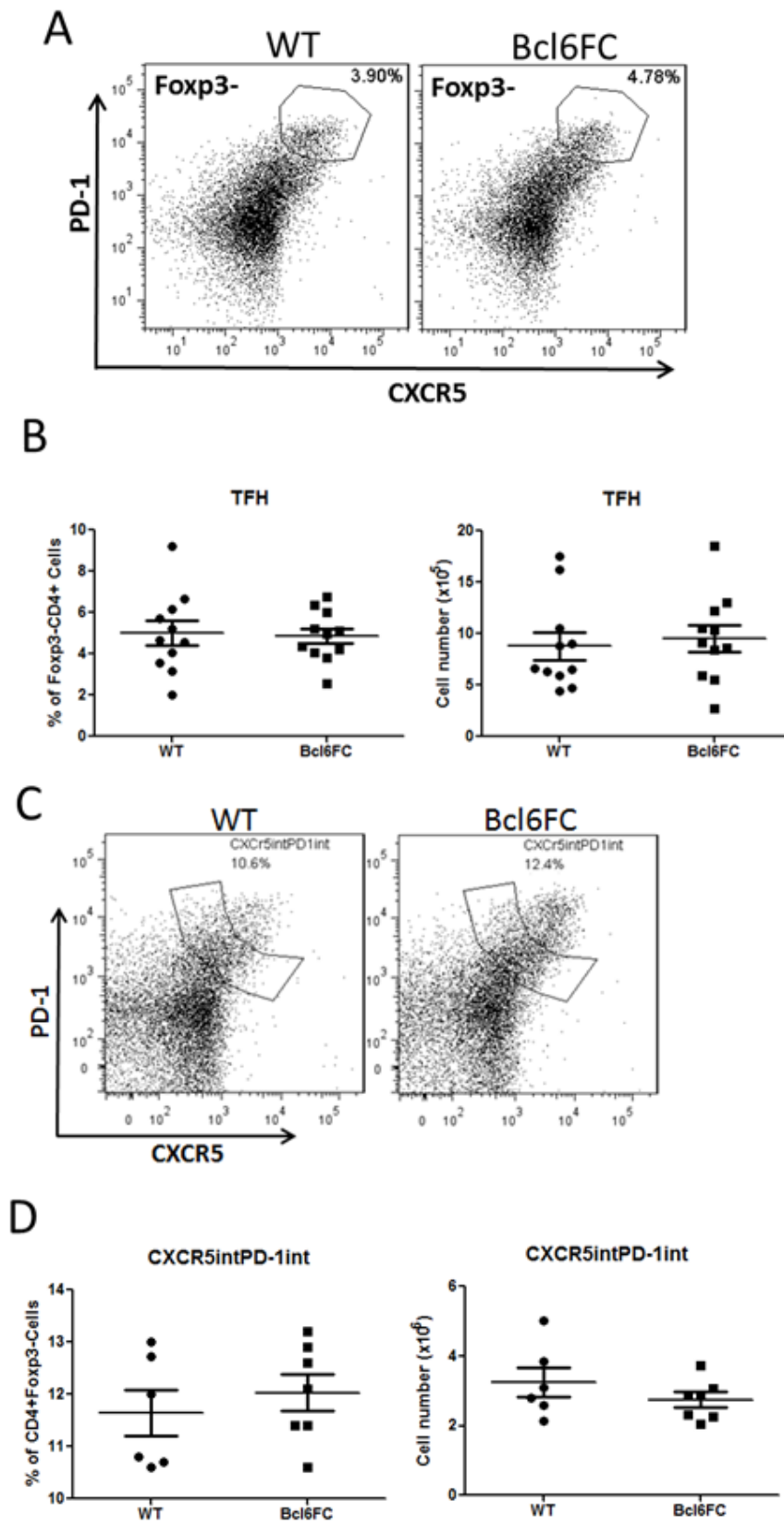


Figure 23. Loss of Bcl6 in Treg cells does not affect pre-TFH and TFH cell differentiation.

Control and Bcl6^{FC} KO mice were immunized with SRBC by i.p. injection. At 10 dpi, spleens were isolated for flow cytometric analysis. (A) TFH cells are defined as Foxp3⁻CXCR5^{hi}PD-1^{hi}. (B) TFH cell percentage in CD4⁺Foxp3⁻ T cells and TFH cell number. (C) The flow plots of pre-TFH cells which are defined as Foxp3⁺CXCR5^{int}PD-1^{int}. (D) pre-TFH cell percentage in CD4⁺Foxp3⁻ T cells and pre-TFH cell number. Graphs show mean +/- SEM, n = 6-7. ** p < 0.01 (student t test). Data are combined from two independent experiments.

3.3.3: Loss of TFR cells does not expand the GC B-cell population but the antibody response is altered.

Although we did not observe that loss of TFR cells led to the increase in TFH-cell numbers, we next wondered whether TFR cells could suppress the GC B cell response. Unexpectedly, 10 days after SRBC immunization, similar sizes of GC B cell populations were formed both in control and Bcl6FC mice (Fig. 24A-B). Similar to our observations with TFH cells, this result of unchanged GC B cell numbers in the absence of TFR cells was observed after multiple types of immune challenge with different antigens and was seen at all time-points following immunization. Since the ultimate outcome of GC responses is the production of antigen-specific antibodies, we next analyzed SRBC-specific IgG and IgM serum antibody titers at 10 dpi by ELISA. Despite the similar TFH- and GC B-cell responses in control and Bcl6FC mice, we found the titers of anti-SRBC-specific IgG were strongly and significantly decreased in Bcl6FC mice, while the titers of anti-SRBC-specific IgA were strongly and significantly increased in Bcl6FC mice (Fig. 24C-D). This pattern of decreased Ag-specific IgG but increased Ag-specific IgA was also observed when the mice were immunized with NP-KLH in Alum (Fig. 25E-F). This indicates that TFR cells are important for promoting high levels of IgG and repressing abnormal IgA responses.

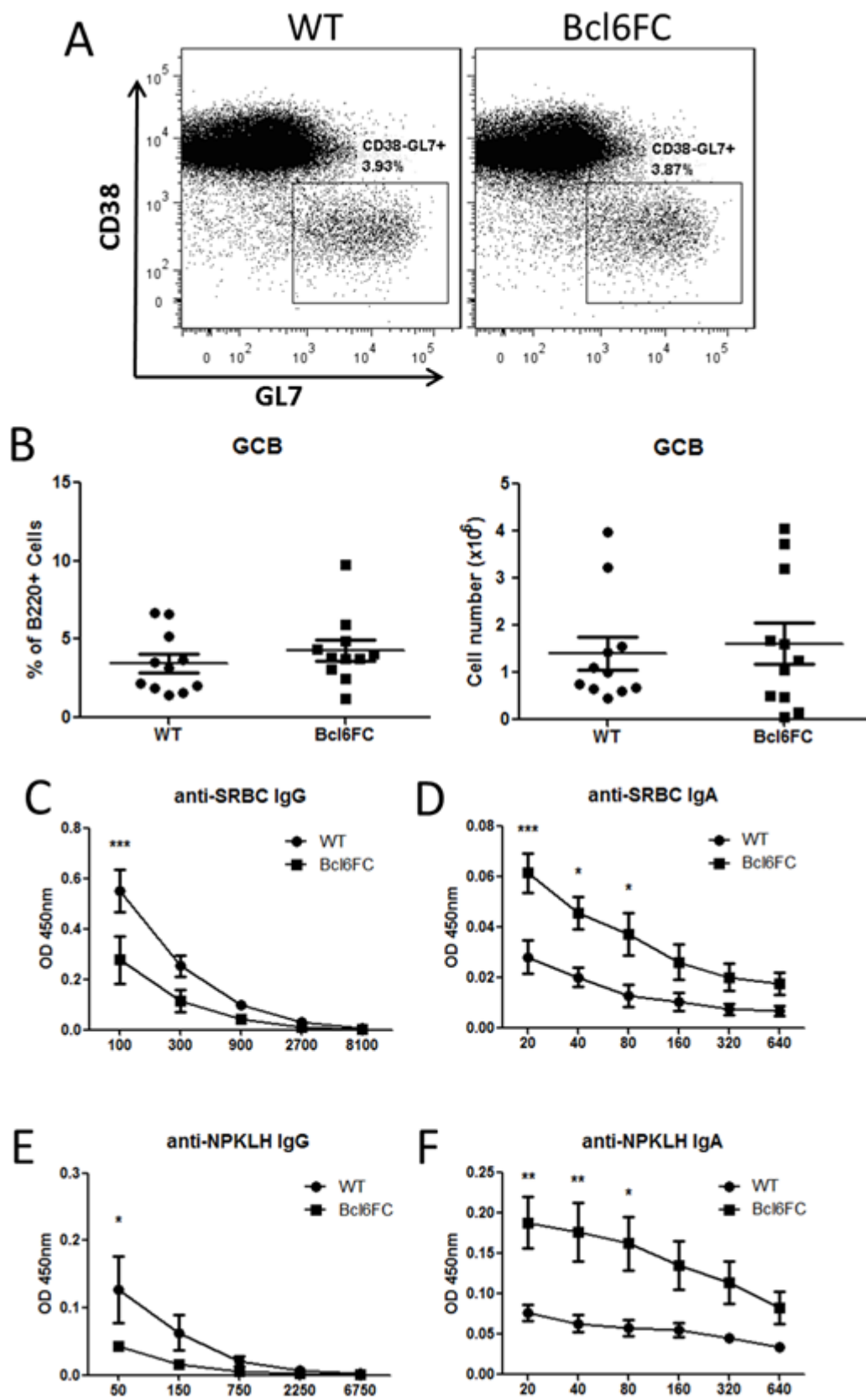


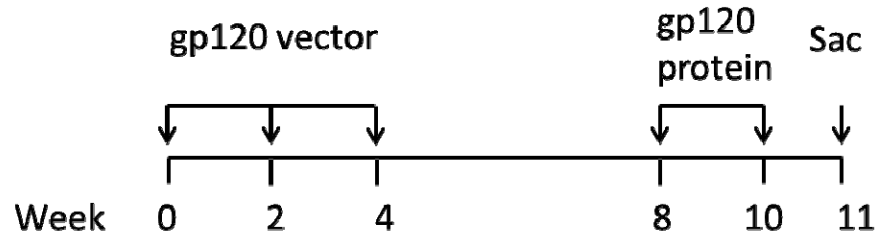
Figure 24. TFR cells do not regulate the size of GC B cell population, but contribute to the generation of antigen-specific IgG antibodies by restraining antigen-specific IgA antibodies.

Control and Bcl6FC KO mice were immunized with SRBC by i.p. injection. Spleens were isolated for flow cytometric analysis at 10 dpi. (A) GC B cells are defined as B220⁺CD38⁻GL7⁺ in flow plot. (B) GC B cell percentage in B220⁺ cells and cell number. Serum samples from control and Bcl6FC mice were also collected. (C) Anti-SRBC IgG, (D) anti-SRBC IgA titers. The X-axis shows the dilution factors. Control and Bcl6FC mice were immunized with NP-KLH in Alum. Serum samples were collected at 25 dpi. (E) Anti-NPKLH IgG, (F) anti-NPKLH IgA titers. The X-axis shows the dilution factors. Graphs show mean \pm SEM, (A-B) n = 6-7, (C-D) n = 4, (E-F) n = 4-6. **p < 0.01, *** p < 0.001 (student t test for B, two-way ANOVA for C-F). For A-B, data are combined from two independent experiments. For C-F, data are representative of three independent experiments with similar results.

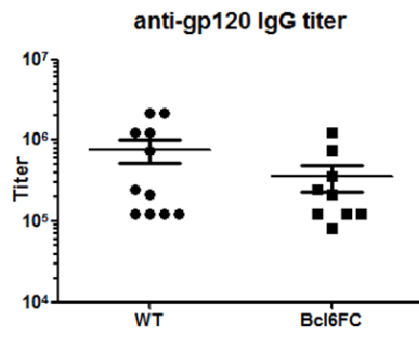
3.3.4: Loss of TFR cells decreases the avidity of antigen-specific antibodies in an HIV immunization model.

Generation of high-affinity, protective antibodies is one of the goals of an effective vaccine. We therefore sought to determine whether TFR cells have an impact on the GC and antigen-specific antibody responses in a vaccination setting. We used a well-characterized DNA prime-protein boost vaccine strategy [110, 111] to raise an antibody response against the HIV-1 envelope glycoprotein gp120 in control and Bcl6FC mice. As shown in Fig. 25A, control and Bcl6FC mice were injected i.m. with gp120-encoding DNA 3 times, 2 wk apart, for priming. 4 wk later, mice received two gp120 protein booster immunizations via i.m. injection, 2 wk apart. One wk after the final injection, mice were sacrificed. In this repeated challenge model, we again did not see significant changes in the size of TFH- or GC B-cell populations in Bcl6FC mice compared to control mice (data not shown).

A



B



C

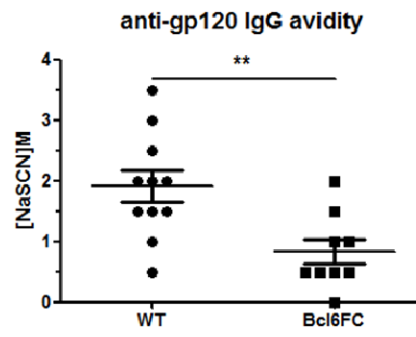


Figure 25. Decreased antibody avidity in the absence of TFR cells in the DNA prime-protein boost gp120 vaccine model.

(A) Experimental setup of gp120 DNA prime and protein boost immunization. Control and Bcl6FC mice were primed i.m. with gp120-encoding DNA 3 times, 2 wk apart. 4 wk later they were given 2 booster injections of gp120 protein, 2 wk apart. Spleen and serum samples from control and Bcl6FC mice were collected one week after the final booster. (B) Serum anti-gp120 specific IgG titers measured by ELISA and (C) avidity of gp120-specific IgG antibodies measured by NaSCN displacement method are shown. Graphs show mean \pm SEM, n = 9-11. **p < 0.01 (student t test). Data are combined from two independent experiments.

Unlike SRBC immunization, where Bcl6FC mice had a dramatic decrease of anti-SRBC IgG titers, anti-gp120 IgG titers were similar in the serum from control and Bcl6FC mice (Fig. 25B). Because of the high titers of IgG produced in this model, we could assess the avidity of anti-gp120 IgG antibodies using a chaotropic reagent (NaSCN) displacement method (Fig. 25C). We observed a significant decrease in the avidity of anti-gp120 IgG antibodies in the Bcl6FC mice. Thus, TFR cells have a positive role in maintaining anti-gp120 IgG antibody avidity in a multi-prime and multi-boost vaccine model. As with other antigens, we saw enhanced levels of anti-gp120 IgA (Fig. 26).

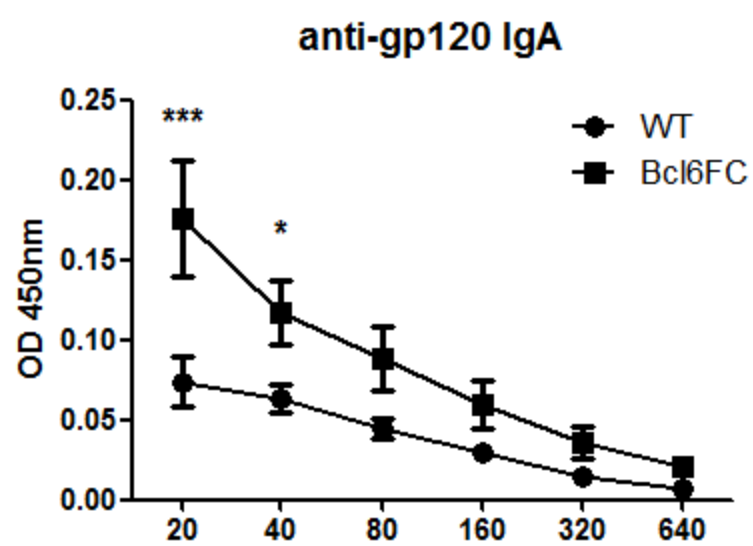


Figure 26. TFR cells regulate the production of anti-gp120 IgA antibodies in the DNA prime-protein boost gp120 vaccine model.

Gp120 immunization is described in Fig. 25. Serum anti-gp120 specific IgA titers measured by ELISA are shown. Graph shows mean \pm SEM, n= 4-6.

**p < 0.01 (student t test). Data are from one experiment.

3.3.5: TFR cells regulate anti-dsDNA IgA antibody production in pristine induced lupus model.

A major function of Tregs is the maintenance of self-tolerance, in order to prevent autoimmune diseases such as SLE. SLE patients exhibit high titers of pathogenic auto-antibodies and they usually have impaired Tregs, both in number and function [120, 121]. We hypothesized that loss of TFR cells could make Bcl6FC mice more susceptible to SLE due to defective control of auto-antibody production. Control and Bcl6FC mice were injected i.p. with pristane to induce a lupus-like syndrome [122, 123]. Four months after treatment, both control and Bcl6FC mice were healthy and without any sign of arthritis, a symptom sometimes seen in the pristane model. As with other immune challenge models, we did not find any changes in TFH- or GC B-cell populations between control and Bcl6FC mice (data not shown). Control and Bcl6FC mice had similar titers of anti-dsDNA IgM and IgG antibodies in serum (Fig. 27A-B). However, we found significantly elevated anti-dsDNA IgA serum antibodies in Bcl6FC mice (Fig. 27C). Thus, in the pristane-induced lupus model, TFR cells repress production of anti-dsDNA IgA, but not other isotypes of anti-dsDNA auto-antibodies.

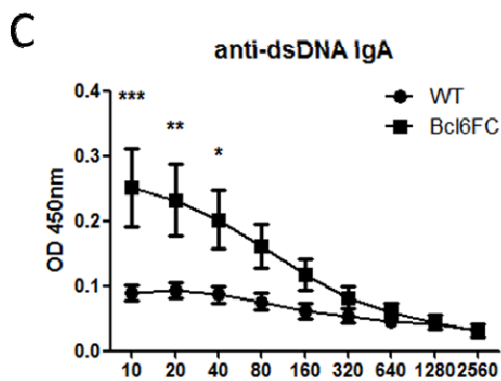
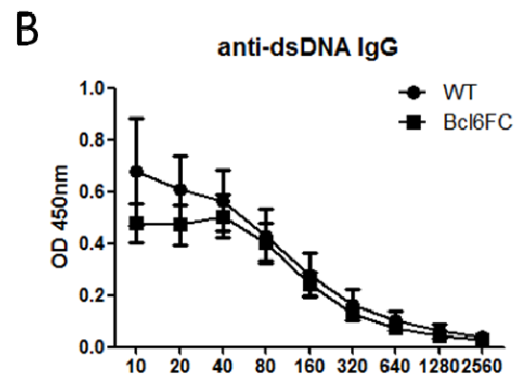
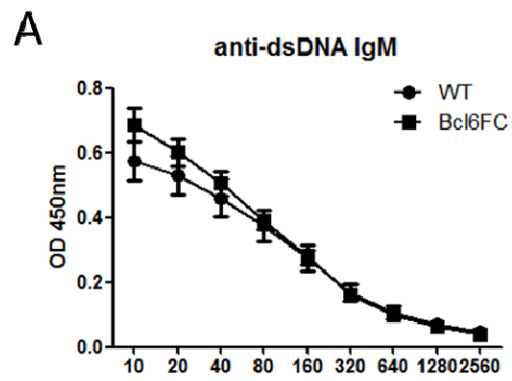


Figure 27. TFR cells regulate the production of anti-dsDNA IgA antibodies in Pristane-induced lupus model.

Four months after single 0.5 ml pristane i.p. injection, serum samples were collected from control and Bcl6FC mice for anti-dsDNA antibody detection by ELISA. (A) Anti-dsDNA IgM, (B) anti-dsDNA IgG and (C) anti-dsDNA IgA titers are shown. The X-axis labels are dilution factors. Graphs show mean \pm SEM, $n = 6-7$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (two-way ANOVA). Data are combined from two independent experiments.

TFR cells regulate cytokine production by TFH cells. Cytokines produced by TFH cells can promote GC B-cell proliferation and survival. Therefore we checked the production of cytokines (IFN- γ , IL-4, IL-10 and IL-21) known to be expressed by TFH cells in splenic PD-1^{hi}, PD-1^{int} and PD-1^{neg} CD4⁺ T cells described previously [112]. Control and Bcl6FC mice were analyzed 10 days after SRBC immunization (Fig. 28A). As shown in Fig. 28B, PD-1^{hi} TFH cells in Bcl6FC mice expressed significantly higher levels of several cytokines: IFN- γ , IL-10 and IL-21. There was no difference in IFN- γ and IL-21 expression by PD-1^{int} and PD-1^{neg} CD4⁺ T cells between control and Bcl6FC mice, however T cells generally produced more IL-10 in Bcl6FC mice (Fig. 28C-D). We observed a very similar cytokine phenotype in the TFH cells in the gp120 DNA prime protein boost model (data not shown). Taken together, these data show TFR cells specifically regulated IFN- γ and IL-21 production by TFH cells, with little effect on cytokine production by PD-1^{int} or PD-1^{neg}CD4⁺ T cells.

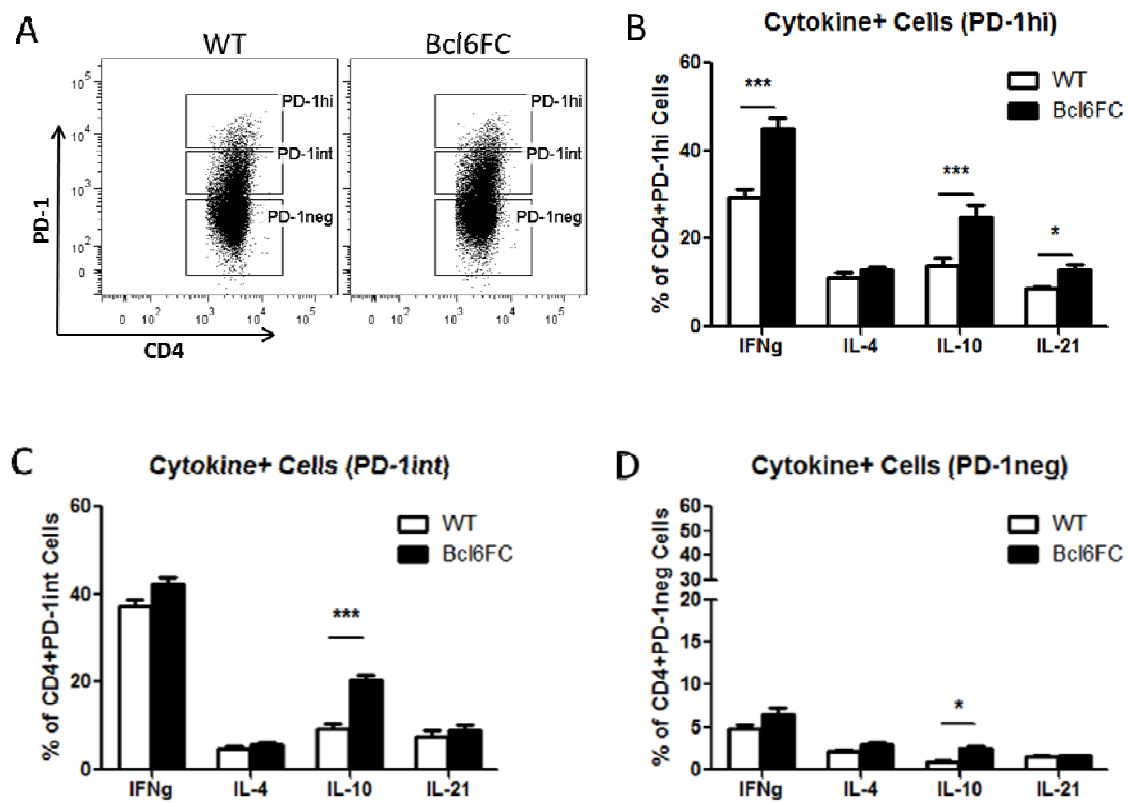


Figure 28. TFR cells regulate IFN- γ , IL-10 and IL-21 production in TFH cells.

(A) PD-1^{hi}, PD-1^{int} and PD-1^{neg} CD4⁺ T cells gates. TFH cells are defined as CD4⁺PD-1^{hi} cells. Percentages of cytokine-producing (IFN- γ , IL-4, IL-10 and IL-21) in CD4⁺PD-1^{hi}TFH (B), CD4⁺PD-1^{int}T (C) and CD4⁺PD-1^{neg}T cells (D) from control and Bcl6FC mice 10 days after SRBC immunization measured by ICS are shown. Graphs show mean \pm SEM, n = 6-7. *p < 0.05, *** p < 0.001 (student t test). Data are combined from two independent experiments.

3.4: Discussion.

Here we have further characterized the phenotype and function of TFR cells. We show that TFR cells express much lower level of CD25 and higher level of inhibitory molecule TIGIT than non-TFR Tregs. We have used the Bcl6FC mouse strain to specifically delete Bcl6 in Tregs to analyze the regulation of TFH cells and the GC reaction by TFR cells. Unexpectedly, TFR cells do not regulate population numbers of TFH or GC B cells, but regulate the normal antibody response following immune challenge, as shown with several models. Strikingly, TFR cells can suppress the generation of IgA anti-dsDNA antibodies in a pristane-induced autoimmune setting. Finally, TFH cells consistently secrete more cytokines in the absence of TFR cells, and we propose that this alters the cytokine milieu and affects the selection of GC B cells, leading to an abnormal antibody response.

The Bcl6FC mouse model is much more amenable to future in-depth experimentation than currently published approaches to specifically analyze TFR-cell function. These mice can be bred in large numbers and will be useful to assess the role of TFR cells in a wide variety of different immune conditions, from infection to autoimmunity. The Bcl6FC model stands in contrast to other approaches to analyze TFR cells in which total Tregs were depleted after immunization [60, 61]. Total loss of Tregs can lead to wide-ranging immune activation that could impact the GC response by indirect means than by TFR cells, such as by affecting early T cell proliferation before TFH differentiation has occurred. Another approach used to study TFR-cell function is to transfer T

helper cells plus or minus TFR cells into T cell-deficient animals, and analyze the antibody responses after immunization [60, 62, 64]. However, this approach has limitations in performing large numbers of such reconstitutions, and it is not clear whether such a reconstituted GC response is physiological. Another study deleted NFAT2 in Tregs or total CD4⁺ T cells to study the function of TFR cells [104]. However the TFR-cell deletion efficiency of this model was only about 50% and this study primarily focused on the up-regulation of CXCR5 of TFR cells by NFAT2, without characterizing a more general Treg defect due to the deletion of NFAT2. Thus, the Bcl6^{FC} mouse model shown here has much promise for further studies into TFR cell function.

CD25 is one of the three subunits of IL-2 receptor whose signaling is critical for Treg differentiation, maintenance and survival [124, 125]. The down-regulated IL-2 signaling in TFR cells due to their low expression of CD25 may explain the high rate of cell death of TFR cells (Fig. 22). Of note, the remaining few TFR cells in Bcl6^{FC} mice have significantly higher levels of cell death than WT TFR cells (Fig. 22). This indicates that Bcl6 may stabilize the survival of TFR cells by counteracting the stress caused by a decreased level of IL-2 signaling. Importantly, unlike non-TFR Tregs, CD25 is not a reliable surface marker for detecting TFR cells.

As noted in the introduction, other studies observed expanded TFH- and GC B-cell responses in the absence of TFR cells. We propose that this is because in these earlier studies, total Tregs were depleted, which led to enhanced early TFH priming or stronger responses during an ongoing GC

response. One possibility with our Bcl6^{FC} mouse model is that the Tregs cannot up-regulate Bcl6 and thus are displaced outside the B cell follicle. In theory, these displaced Tregs could more strongly suppress developing TFH responses on the edge of the B cell follicle, and this could then mask the effect of loss of TFR cells on TFH cells within the GC, and balance out the stronger TFH/GC responses that would be otherwise seen without TFR cells. While this is a possibility, we show in Fig. 28C that PD1- and CXCR5-intermediate Foxp3⁺ T cells, which can be considered as early- or pre-TFH cells, do not have a decreased expression of cytokines as evidence of increased Treg suppression. Also, in the Bcl6^{FC} mice, there is not a decreased percentage of PD1- and CXCR5-intermediate Foxp3⁺ T cells (early/pre-TFH cells) that would be consistent with increased suppression by Tregs at this stage (Fig. 23). Another possibility is that the few TFR cells left in the Bcl6^{FC} mice are enough to prevent abnormal outgrowth of TFH and GC B cells. While this is hard to rule out completely, we doubt that any other system used previously was able to delete TFR cells more completely than we have here, and further, the few remaining TFR cells in Bcl6^{FC} mice are undergoing increased apoptosis (Fig. 22).

Previous studies on TFR cells led to conflicting results on the precise role of TFR cells in controlling antibody responses [60-62, 105]. In general, previous work was in agreement with the idea that TFR cells suppress the size of the GC response. However, previous studies came to different conclusions on the effect of TFR cells on the antibody response. These differing results reflected the systems used to analyze TFR function, which had limitations, as discussed

above. In our study, we used multiple immunization models, immunization with SRBC, NP-KLH/Alum, HIV gp120 DNA prime protein boost vaccination and pristane treatment. In none of these models, did loss of TFR cells lead to an increase of GC B-cell populations at any of the time-points we examined. Unexpectedly, we observed a decrease in Ag-specific IgG production and an increase in Ag-specific IgA production in the Bcl6FC mice, showing a novel role for TFR cells in controlling isotype switching of antibodies. We currently do not know whether in the absence of TFR cells, there is a deviation of the normal IgG response to an IgA response, or whether there are separate defects causing these opposite effects on Ig isotype. In general, the decrease in IgG titer is stronger than the increase in IgA titer, indicating that there is not a simple compensating switch from IgG switching to IgA. Likely, the altered cytokine expression we observe from TFH cells from Bcl6FC mice contributes to the decreased IgG and increased IgA. Answering this question will require a great deal of additional research.

In the HIV-1 gp120 model [110, 111], we observed a decrease in the affinity of anti-gp120 antibodies. One possible explanation for this result, as pointed out in Linterman et al [61], is the expansion of non-gp120-specific or low-affinity Ag-specific GC B cells in the absence of TFR cells. In this model, Bcl6FC mice develop normal titers of anti-gp120 IgG antibodies but with significantly decreased avidity. Probably due to the long term repeated challenge nature of this vaccination strategy, the Bcl6FC mice were able to produce normal titers of antibody but still could not produce highest affinity antibodies due to abnormal

GC B cell selection in the absence of TFR cells. These findings suggest that monitoring TFR cells during the course of vaccination may help predict vaccine efficacy in producing high-affinity antibodies.

TIGIT is an important co-inhibitory molecule on Tregs for controlling pro-inflammatory Th1 and Th17 cells [117]. We demonstrate here that TFR cells express high levels of TIGIT. In the absence of TFR cells, therefore there is a lack of TIGIT-mediated immune suppression from TFR cells in GCs in Bcl6FC mice. Accordingly, we observed increases in production of IFN- γ , a Th1 cytokine, and IL-21, a Th17 or TFH cytokine, by TFH cells in these mice after SRBC immunization or gp120 vaccination. TFH cells themselves express multiple negative immune checkpoint regulators such as, PD-1, BTLA and TIGIT [65, 115, 116], which may relate to why we did not find any abnormality in the size of TFH cell population. Competition for IL-21, a known B cell growth factor, could enhance survival of high-affinity clones throughout the GC response [126, 127]. The increase of IL-21 production by TFH cells in GCs in Bcl6FC mice could provide an enhanced survival signal to non-antigen-specific or low-affinity B cell clones, leading to the decrease in titers of anti-SRBC IgG antibodies and in the avidity of anti-gp120 IgG antibodies.

Tregs are important for the maintenance of the immunological tolerance and self-/non-self-discrimination [125]. One of the hallmarks of SLE is the generation of anti-dsDNA auto-antibodies in which the GC response plays an important role [128]. The discovery of TFR cells gives us a great opportunity to better understand the regulation of GC response in SLE. We used a pristane-

induced lupus model with our Bcl6FC mice. Either the genetic background of our mice (C57Bl/6) is not ideal for pristane treatment [129] or the 4 month incubation period after pristane treatment was not long enough, since neither control mice nor Bcl6FC mice developed severe disease symptoms. However the GC B cell response and antibodies against dsDNA did arise from pristane treatment. We observed a significant increase of anti-dsDNA IgA titer in the absence of TFR cells indicating a critical role of TFR cells in controlling autoimmune GC response in SLE. The increase in production of IFN- γ and IL-21 by TFH cells in Bcl6FC mice could be an additional reason for elevated IgA anti-dsDNA antibody titer, since both IFN- γ and IL-21 are involved the generation of auto-antibodies [130, 131]. In the clinic, an elevated level of IgA anti-dsDNA antibodies is associated with kidney and joint abnormalities in SLE patients [132, 133]. Regulation of the GC response by the TFR cells in SLE patients or other autoimmune diseases is an important area for future investigation.

Overall, our study helps to expand on, clarify and reconcile some of the previous findings on TFR cells. We show TFR cells can promote normal levels and types of antibody responses against foreign antigens, help maintain antibody affinity and regulate generation of anti-self antigen antibodies in autoimmune responses. Our TFR cell-specific deletion mouse model should be further used for understanding the role of TFR cells in vaccination and in controlling pathogenic antibody responses.

Chapter 4 - STAT3 is important for Follicular Regulatory T cell differentiation

4.1: Introduction.

To provide host protection against pathogens, CD4⁺ T cells differentiate into several distinct lineages that confer specific effector functions. For instance, viral or intracellular pathogens typically induce protective Th1 responses, while helminth parasites induce Th2 responses. TFH cells are a T helper cell lineage whose major function is to help B cells form GCs and produce high-affinity antibodies [102, 103]. Commitment of naive T cells to these different effector lineages is highly dependent on the cytokines present in the immunological milieu. Cytokine receptor signaling activates specific transcription factor pathways. STAT4-Tbet, STAT6-GATA3 and STAT3-ROR γ promote Th1, Th2 and Th17 cell differentiation respectively [134]. TFH cell differentiation is driven by the transcriptional repressor Bcl6, which can be induced downstream of STAT1, STAT3 or STAT4 signaling [56, 57, 72, 76, 135]. TFH cells control the initiation as well as the outcome of the GC B cell response. While TFH cells are critical for the proper production of antibodies, the over-production of TFH cells can lead to autoimmunity, since TFH cells can help B cells to produce self-reactive antibodies [69-71]. Thus, the proper regulation of TFH and GC B cell responses is essential both for normal immune function and for preventing autoimmune disease.

Treg cells repress the activation, proliferation and function of effector T helper cells during immune responses and are critical to restrain autoimmune responses. In both humans and mice, deficiency of Foxp3 results in a fatal multi-organ autoimmune disorder. Specifically, Foxp3-mutant Scurfy mice have uncontrolled T cell proliferation and drastically elevated inflammation mediated by Th1, Th2 and Th17 responses as well as exacerbated production of autoreactive antibodies [136, 137]. Previous studies showed multiple subsets of Treg cells possess unique regulatory properties to repress corresponding pathological immune responses. For example, Tbet controls Treg functions during Th1 mediated inflammation [138]. IRF-4 in Treg cells is important for regulation of Th2 responses [139]. Deletion of STAT3 in Treg cells results in dysregulation of Th17 responses [89]. Recently, a subpopulation of Treg cells in the GCs was discovered that appears to act as suppressors of TFH and GC B cells. These regulatory follicular T cells or “TFR” cells depend on Bcl6 for differentiation and chemokine receptor CXCR5 to localize to the B cell follicle [60-62].

Ding et al revealed that IL-21, which activates STAT3 signaling, can suppress TFR cell differentiation in BXD2 autoimmune mice [140]. In contrast, IL-6-STAT3 and IL-21-STAT3 signaling promote TFH cell differentiation by induction of Bcl6 expression [56, 72, 141]. It is therefore important to determine whether STAT3 promotes or inhibits TFR cell development. Treg cells tend to adopt part of the transcriptional program of the specific T helper cell subsets they regulate. Therefore, the requirement for STAT3 in Treg cells to suppress TFH

cell mediated humoral responses is of particular interest. Previous studies show TFR and TFH cells derived from Treg cells [142] or Th17 cells [143] in PPs promote the diversification and selection of IgA antibodies which in turn maintain the bacterial diversity at the mucosal barrier in the gut. However, in Treg cells, the role of STAT3 in the regulation of gut microbiota is not clear.

Here, we found that STAT3 is essential for TFR cell differentiation both in spleen following antigen immunization and in PPs. Deletion of STAT3 causes a much more severe defect in TFR cell differentiation than in TFH cell differentiation. Interestingly, loss of TFR cells in STAT3^{fl/fl}Foxp3^{cre} mice has no effect on the size of TFH or GC B cell populations after immunization, but does lead to increased production of antigen-specific IgG antibodies.

4.2: Materials and Methods.

Mice and immunizations. STAT3^{fl/fl} mice were crossed with CD4-cre transgenic mice or Foxp3-YFP-cre mice. Bcl6^{fl/fl} mice were crossed with Foxp3-YFP-cre mice. Foxp3-YFP-cre mice and BoyJ mice were purchased from The Jackson Laboratory (JAX). For STAT3KO mice, littermate STAT3^{fl/fl} mice were used as WT control. For STAT3^{fl/fl}Foxp3^{cre} (STAT3FC) or Bcl6FC mice, littermate Foxp3^{cre} mice were used as WT control. Mice were bred under specific pathogen-free conditions at the laboratory animal facility at IUSM and were handled according to protocols approved by the IUSM Animal Care and use Committee. For SRBC immunization, mice were i.p. injected with 1 x 10⁹ SRBCs (Rockland Immunochemicals) and were sacrificed at the indicated day.

Flow cytometry reagents. Anti-CXCR5 (2G8) and GL7 (GL7) antibodies were from BD Biosciences. Fixable viability dye, anti-CD38 and anti-Foxp3 (FJK-16s) antibodies were from eBioscience. Anti-CD4 (GK1.5), anti-B220 (RA3-6B2), anti-CD45.1 (A20) and anti-PD-1 (29F.1A12) were from Biolegend.

Cell staining for flow cytometry. After red blood cell lysis, total spleen cells were incubated with anti-mouse CD16/CD32 (BioXcell) for 5 minutes at room temperature, followed by surface staining for the indicated markers. For intracellular transcription factor staining, after surface markers were stained, cells were fixed and stained with antibodies against transcription factors by following the Foxp3 fixation kit (eBioscience) instructions. Cell events were collected on an LSRII flow cytometer (Becton Dickinson).

Peyer's patch isolation. PPs were cut using scissors from the small intestine and incubated for 10 mins at 37 °C in PBS containing 1% FBS, 4mM EDTA and 15 mM HEPES (PH 7.2). PPs were washed twice, with vigorous vortexing before spinning, in PBS. Soluble cell debris in supernatants was removed after centrifugation. Isolated PPs were broken apart between two frosted glass microscope slides to generate single cell suspension for flow cytometry staining or ICS described above.

Bone marrow chimeras. Recipient CD45.1⁺ BoyJ mice were lethally irradiated with 1,100 Rad and reconstituted with 2x10⁶ a mixture of nucleated bone marrow cells from either CD45.2⁺ WT mice plus CD45.1⁺ BoyJ mice, or CD45.2⁺ STAT3KO mice plus CD45.1⁺ BoyJ mice by i.v. injection. Chimeric mice were immunized with SRBC approximately 12 weeks after reconstitution.

Antibody titer analysis. Antibody titers of SRBC-specific in serum were measured by ELISA, as previously reported. Briefly, 96 well Nunc-Immuno plates (Sigma) were coated with SRBC membrane protein overnight at 4 °C. Wells were blocked with 10% FCS and diluted serum was added and incubated at room temperature for 2 h. A peroxidase-conjugated anti-mouse IgG was used as secondary antibody.

16s rRNA sequencing and data analysis. Cecum samples were stored at -80 until use. DNA was purified with PowerFecal DNA isolation kit (MO BIO) according with manufacturer's instructions. Sample sequencing and data analysis were performed at LC Sciences. Briefly, DNA samples were amplified using V3-V4 region primers (U341F, 533R) targeting bacterial 16s rRNA genes. Sequencing was carried out with an Illumina MiSeq sequencer. Sequencing data were processed and analyzed with QIIME.

Statistical Analysis. All data analysis was done using Prism Graphpad software. Unless otherwise stated, Student t test or ANOVA with Tukey post hoc analysis were used. Only significant differences ($p < 0.05$) are indicated in figures.

4.3: Results.

4.3.1: STAT3 deficiency in T cells results in a decrease of TFH cells and a block of TFR cell differentiation.

To test whether STAT3 is required for the differentiation of TFR cells, we immunized control and STAT3KO mice with SRBC and found that loss of STAT3

severely blocked the differentiation of $\text{Foxp3}^+\text{CXCR5}^{\text{hi}}\text{PD1}^{\text{hi}}$ TFR cells (Fig. 29A, C). Consistent with our previous results [112], in STAT3KO mice, deletion of STAT3 leads to a decrease of $\text{Foxp3}^+\text{CXCR5}^{\text{hi}}\text{PD1}^{\text{hi}}$ TFH cell in cell number, but not in percentage (Fig. 29B, D). Both TFH cells and TFR cells were significantly decreased, however TFR cells were almost gone whereas 60% of TFH cells still remained in the absence of STAT3 in T cells. We calculated the relative decrease levels for TFH and TFR cells due to deletion of STAT3, and found that the proportional loss of TFR cells is much higher than for TFH cells (Fig. 29E). These results suggest that STAT3 in CD4 T cells are required for normal TFH and TFR cell differentiation.

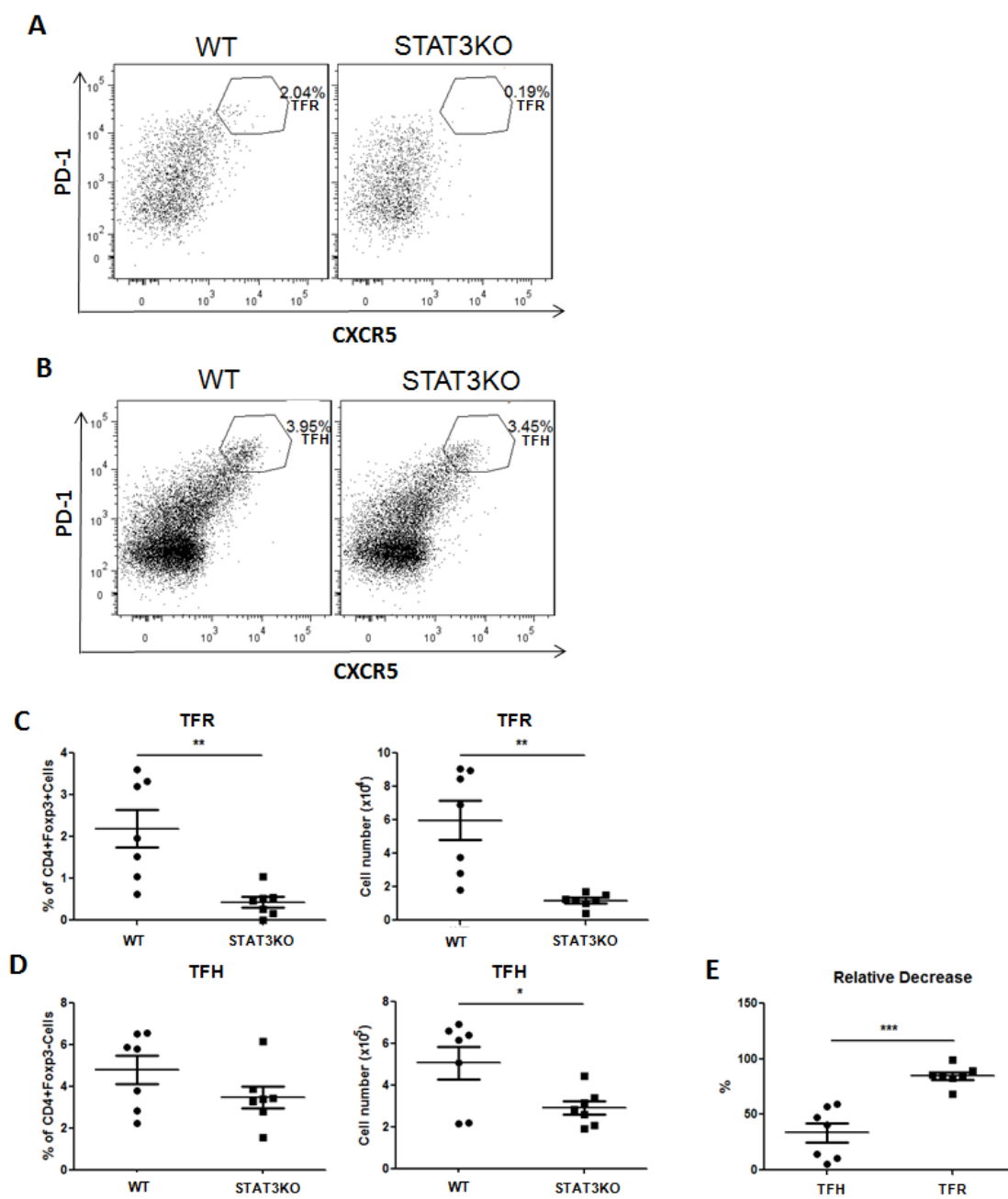


Figure 29. STAT3KO mice have impaired TFH and TFR cell responses following SRBC immunization.

Control and STAT3KO mice were immunized with SRBC via i.p. injection. Spleens were isolated for flow cytometric analysis at 7 dpi. (A and B) Flow cytometry plots of TFR cells which are CD4⁺Foxp3⁺CXCR5^{hi}PD-1^{hi} (A) and TFH cells which are CD4⁺Foxp3⁻CXCR5^{hi}PD-1^{hi} (B). (C) TFR cells percentages (left) and numbers (right) gated as in (A). (D) TFH cells percentages (left) and numbers (right) gated as in (B). (E) Analysis of data in (C and D) showing relative decrease of TFH cells and TFR cells in STAT3KO mice compared to the ones in control mice. Relative decrease = $[(\% \text{ CXCR5}^{\text{hi}}\text{PD-1}^{\text{hi}} \text{ cells in control mice} - \% \text{ CXCR5}^{\text{hi}}\text{PD-1}^{\text{hi}} \text{ cells in STAT3KO mice}) / \% \text{ CXCR5}^{\text{hi}}\text{PD-1}^{\text{hi}} \text{ cells in control mice}] \times 100$. Graphs show mean \pm SEM, n = 7. *p < 0.05, **p < 0.01, *** p < 0.001 (student t test). Data are combined from two independent experiments.

4.3.2: The differentiation defect in TFR cell is Treg cell intrinsic.

The population of GC B cells was dramatically decreased in STAT3KO mice [112] and B cells are important for TFR cell differentiation and maintenance [144]. The loss of TFR cells in STAT3KO mice could be due to either lack of GC B cell support or loss of intrinsic STAT3 function. To investigate these possibilities, we generated mixed BM chimeras in which we transferred CD45.1⁻ BM donor cells from WT or STAT3KO mice mixed with CD45.1⁺ BM donor cells from BoyJ mice into lethally irradiated CD45.1⁺ BoyJ mice. After 3 months of engraftment, we immunized the chimera mice with SRBC and analyzed spleen cells at 7 days dpi. Both mice received WT BM and STAT3KO BM had similar populations of GC B cells (data not shown). WT donor cells were able to differentiate into TFR and TFH cells, whereas STAT3KO donor cells had a severe defect in TFR and TFH cells (Fig. 30D-E). Importantly, the decrease of TFR cells was much higher than of TFH cells in the chimera mice (Fig. 30F), consistent with the results from the directly immunized WT and STAT3KO mice. These results suggest that TFR cell differentiation intrinsically requires STAT3. Moreover, TFR cell differentiation is more dependent on STAT3 signaling than is TFH cell differentiation.

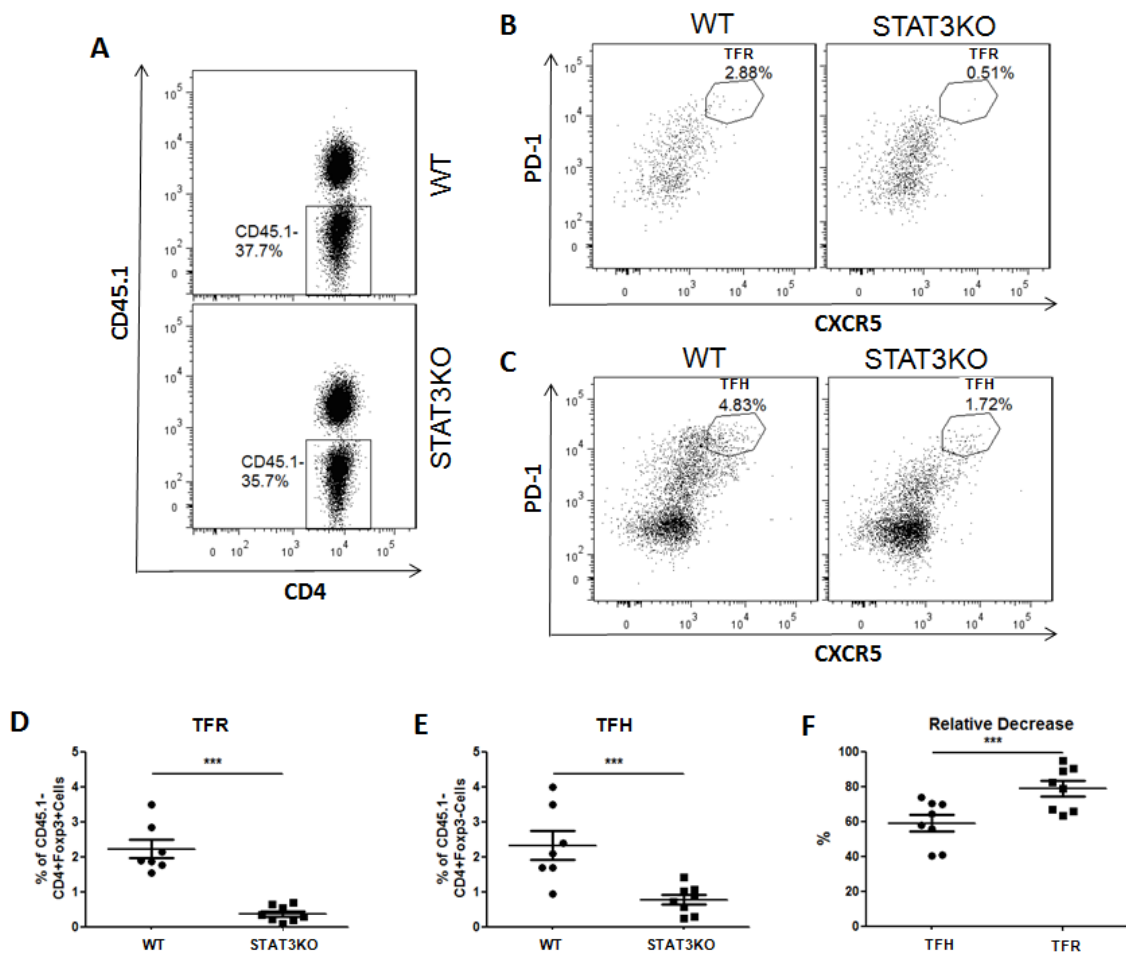


Figure 30. Intrinsic defect of STAT3-deficient TFH and TFR cell differentiation.

CD45.1⁻ bone marrow (BM) cells from WT or STAT3KO mice mixed with CD45.1⁺ BM cells from BoyJ mice were transplanted into lethally irradiated BoyJ mice. Three months later, chimera mice were immunized with SRBC via i.p. injection. Spleens were isolated for flow cytometric analysis at 7 dpi. (A) Flow cytometry plots of splenic CD45.1⁻ cells derived from WT or STAT3KO donor BM. (B) Flow cytometry plots of TFR cells which are CD4⁺Foxp3⁺CXCR5^{hi}PD-1^{hi} generated from WT (left) and STAT3KO (right) donor BM. (C) Flow cytometry plots of TFH cells which are CD4⁺Foxp3⁻CXCR5^{hi}PD-1^{hi} generated from WT (left) and STAT3KO (right) donor BM. (D) TFR and (E) TFH cells percentages gated as in (B) and (C) respectively. (F) Analysis of data in D and E showing relative decrease of TFH cells and TFR cells from STAT3KO donor BM compared to the ones from WT donor BM. Relative decrease = $[(\% \text{ CXCR5}^{\text{hi}}\text{PD-1}^{\text{hi}} \text{ cells from WT BM} - \% \text{ CXCR5}^{\text{hi}}\text{PD-1}^{\text{hi}} \text{ cells from STAT3KO BM}) / \% \text{ CXCR5}^{\text{hi}}\text{PD-1}^{\text{hi}} \text{ cells from WT BM}] \times 100$. Graphs show mean \pm SEM, n = 7-8. *** p < 0.001 (student t test). Data are combined from two independent experiments.

4.3.3: Loss of TFR cells does not lead to an increased TFH cell population in STAT3FC mice.

In order to assess the function role of STAT3 in Treg cells regulating GC response, we created STAT3^{fl/fl}Foxp3^{cre} (STAT3FC) mice, where floxed STAT3 allele is deleted specifically in Foxp3 expressing T cells. In contrast to previous study [89], STAT3FC mice are healthy, fertile, do not display abnormal inflammation and have grossly normal immune cell compartments (data not shown) by at least 5 months of age. We immunized WT and STAT3FC mice with SRBC and analyzed TFH and TFR cells in spleen at 10 dpi. As expected, the differentiation of Foxp3⁺CXCR5^{hi}PD-1^{hi} TFR cells was strongly diminished in STAT3FC mice (Fig. 31A, C). We did not see an increase in the proportion or number of TFH cells in immunized STAT3FC mice (Fig. 31B, D). This result is not unique to SRBC immunization, as STAT3FC mice also had comparable TFH cell population as control mice did at 25 dpi of SRBC immunization as well as KLH-Alum immunization (data not shown). We also found a similar phenotype in Bcl6FC mice, where Bcl6 is specifically deleted in Treg cells [145]. Bcl6FC mice had severely depleted TFR cells, however they had normal TFH cell differentiation in spleen after immunization. Thus, TFR cells are strongly decreased in STAT3FC mice, however loss of TFR cells does not lead to an enlarged TFH cell population.

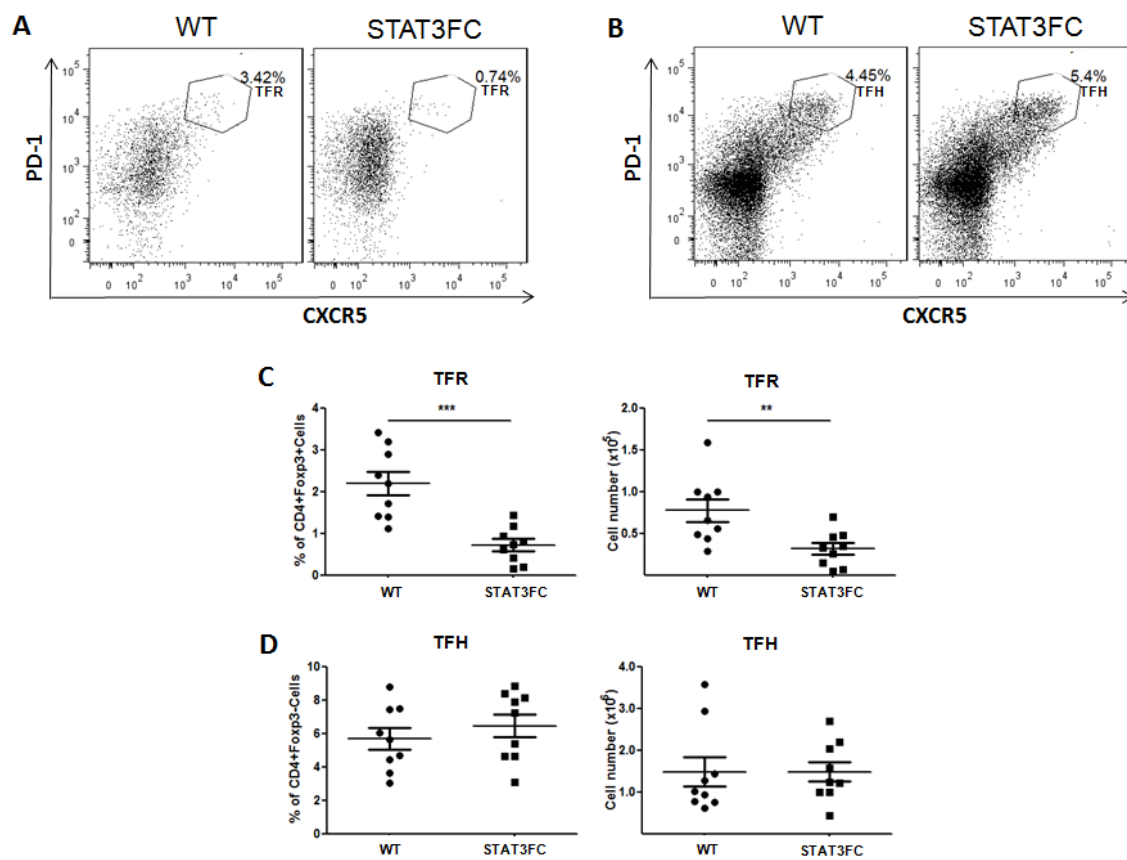


Figure 31. Loss of TFR cells in STAT3FC mice does not affect the size of TFH cell population after SRBC immunization.

Control and STAT3FC mice were immunized with SRBC via i.p. injection. Spleens were isolated for flow cytometric analysis at 10 dpi. (A and B) Flow cytometry plots of TFR cells which are CD4⁺Foxp3⁺CXCR5^{hi}PD-1^{hi} (A) and TFH cells which are CD4⁺Foxp3⁻CXCR5^{hi}PD-1^{hi} (B). (C) TFR cells percentages (left) and numbers (right) gated as in (A). (D) TFH cells percentages (left) and numbers (right) gated as in (B). Graphs show mean +/- SEM, n = 9. ** p < 0.01, *** p < 0.001 (student t test). Data are combined from three independent experiments.

4.3.4: Loss of TFR cells does not lead to an increased GC B cell population in STAT3FC mice but increases the generation of antigen-specific IgG antibodies.

Previous reports show TFR cells can directly inhibit B cell responses [60-62]. We next wondered whether this is the case in our STAT3FC mice. Interestingly, at 10 dpi, similar sizes of B220⁺CD38⁻GL7⁺ GC B cell populations were formed in control and STAT3FC mice (Fig. 32A-B). Similar to our observations with TFH cells, this result of unchanged GC B cell numbers in the absence of TFR cells was observed at 25 dpi for both SRBC and KLH-Alum immunization. This is again consistent with our data from Bcl6FC mice [145]. Since the outcome of GC response is to generate high-affinity antigen-specific antibodies, we next analyzed SRBC-specific IgG serum antibody titers at 10, 15 and 25 dpi by ELISA. We found that, at all three time points, the titers of anti-SRBC IgG antibodies were significantly increased in STAT3FC mice. The magnitude of the titer increase for STAT3FC mice became larger over time and the anti-SRBC IgG antibody titer at 25 dpi showed the largest difference (Fig. 32C). These results indicate that STAT3 is required for Treg/TFR cells to suppress antigen-specific antibody production. We next analyzed the specific subtypes of IgG Abs increased in STAT3FC mice, as well as whether other types of Abs are controlled by STAT3-dependent TFR cells. As shown in Fig. 33, the specific IgG subtypes increased in the absence of STAT3-dependent TFR cells are IgG1 and IgG2b. SRBC-specific IgG2c and IgG3 Abs were detected, but were not increased in STAT3FC mice (Fig. 33). SRBC-specific IgM and IgA

responses were elevated in STAT3FC mice but the increase was not significant (Fig. 33).

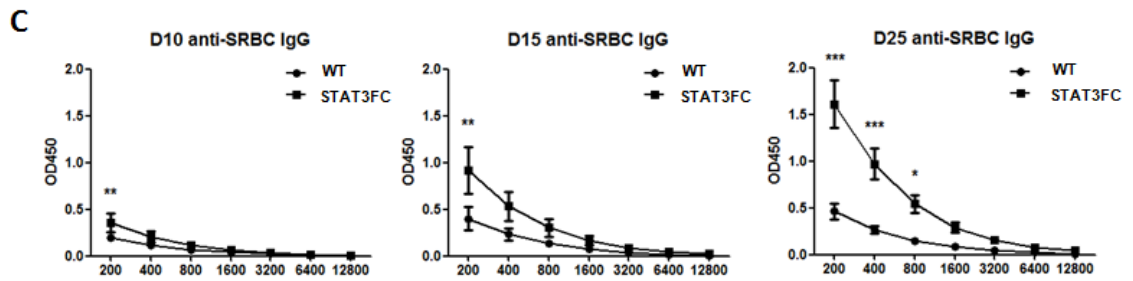
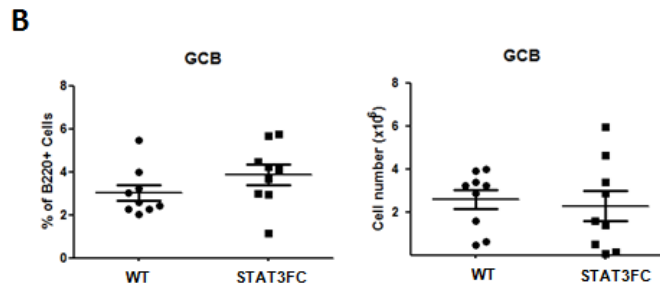
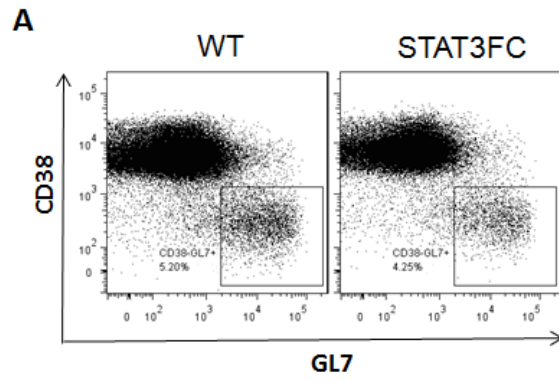


Figure 32. Loss of TFR cells in STAT3FC mice does not affect the size of GC B cell population after SRBC immunization.

Control and STAT3FC mice were immunized with SRBC. Spleens were isolated for flow cytometric analysis at 10 dpi. (A) Flow cytometry plots of GC B cells which are B220⁺CD38⁻GL7⁺. (B) GC B cells percentages (left) and numbers (right) gated as in (A). (C) Serum samples were collected at 10, 15 and 25 dpi following SRBC immunization. Anti-SRBC IgG titers are shown. The X-axis shows the dilution factors. Graphs show mean \pm SEM, (B) n = 9, (C) n = 5. ** p < 0.01, *** p < 0.001 (student t test for B, two-way ANOVA for C). For B, data are combined from three independent experiments. For C, data are representative of three independent experiments with similar results.

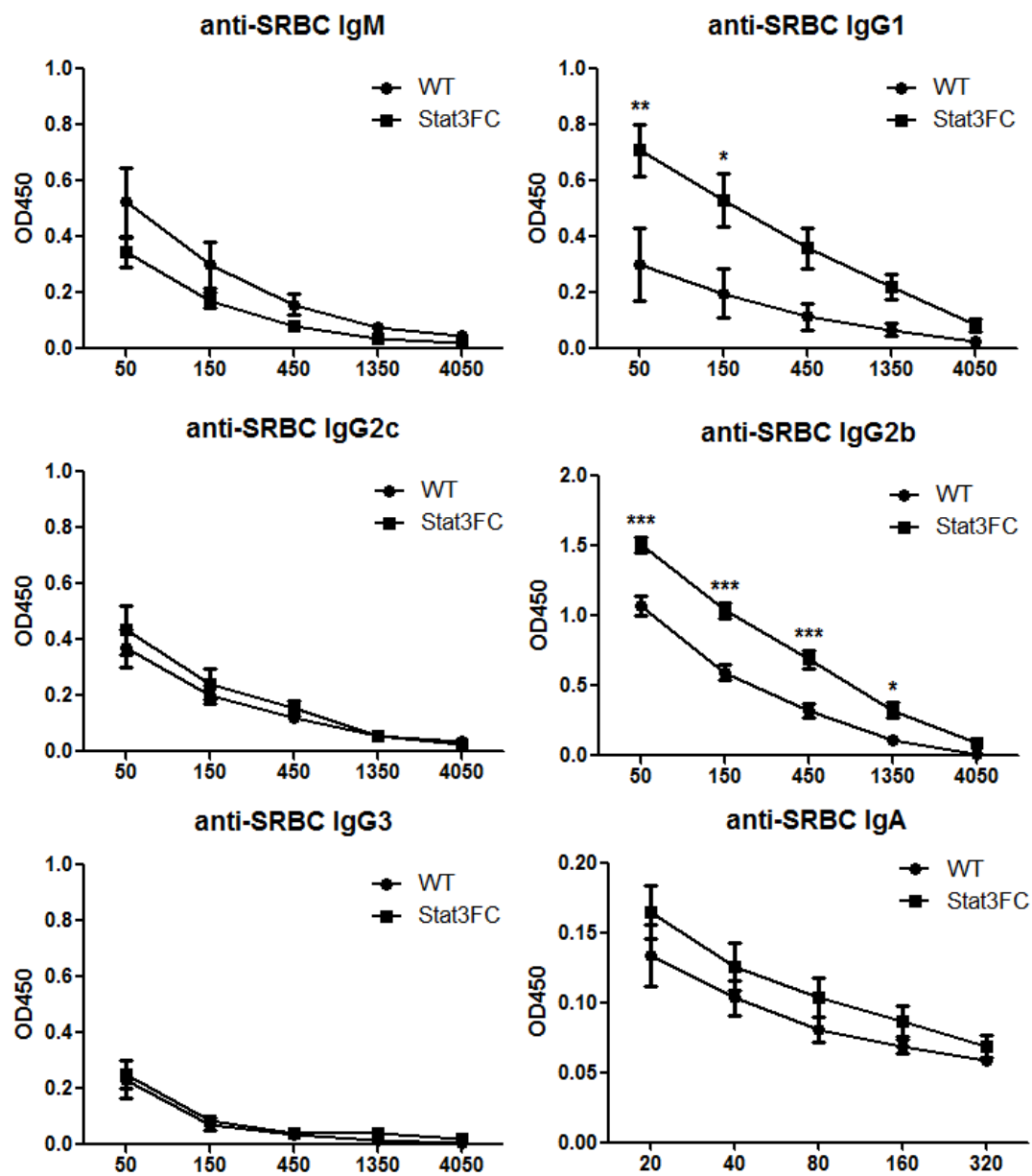


Figure 33. TFR cells regulate the production of anti-SRBC IgG1 and anti-SRBC IgG2b.

Control and STAT3FC mice were immunized with SRBC via i.p. injection. Serum samples were collected at 25 dpi. Anti-SRBC IgM, anti-SRBC IgG1, anti-SRBC IgG2b, anti-SRBC IgG2c, anti-SRBC IgG3 and anti-SRBC IgA titers are shown. The X-axis shows the dilution factors. Graphs show mean \pm SEM, $n = 4$. ** $p < 0.01$, *** $p < 0.001$ (two-way ANOVA). Data are representative of two independent experiments with similar results.

4.3.5: STAT3 function in Treg cells is also required for TFR cell differentiation in Peyer's patches.

A previous study showed that Treg cells can differentiate into TFH cells in PPs in the small intestine [146]. We found that STAT3 is required for both TFH and TFR cell differentiation in spleen (Fig. 29C-D, 30D-E). To further investigate the role of STAT3 in Treg cells in PPs, we examined TFR and TFH cell differentiation in PPs in STAT3^{FC} mice. In STAT3^{FC} mice, TFR cell differentiation in PPs was severely decreased, but TFH cell and GC B cell differentiation was left intact (Fig. 34B-C). These results are unchanged when absolute numbers of these cell populations are analyzed (data not shown). GC B cells switch to IgG1 and IgA at high rates in the PP, and we were able to detect about 20% of PP GC B cells that expressed IgG1 and about 20% that expressed IgA (Fig. 34D-E). However, switching to IgG1 and IgA in the PP was not perturbed by the loss of TFR cells in STAT3^{FC} mice (Fig. 34D-E). Overall, these results indicate that STAT3 is required for TFR formation in the PP, but STAT3-dependent TFR cells do not grossly affect TFH or GC B cell numbers or isotype class switching in the PP. Our data show that TFR cells are almost completely STAT3-dependent for their formation, but that STAT3-dependent TFR cells have little impact on TFH and GC B cell proliferation. Overall, STAT3-dependent TFR cells have relatively subtle roles in fine-tuning the GC and Ab response.

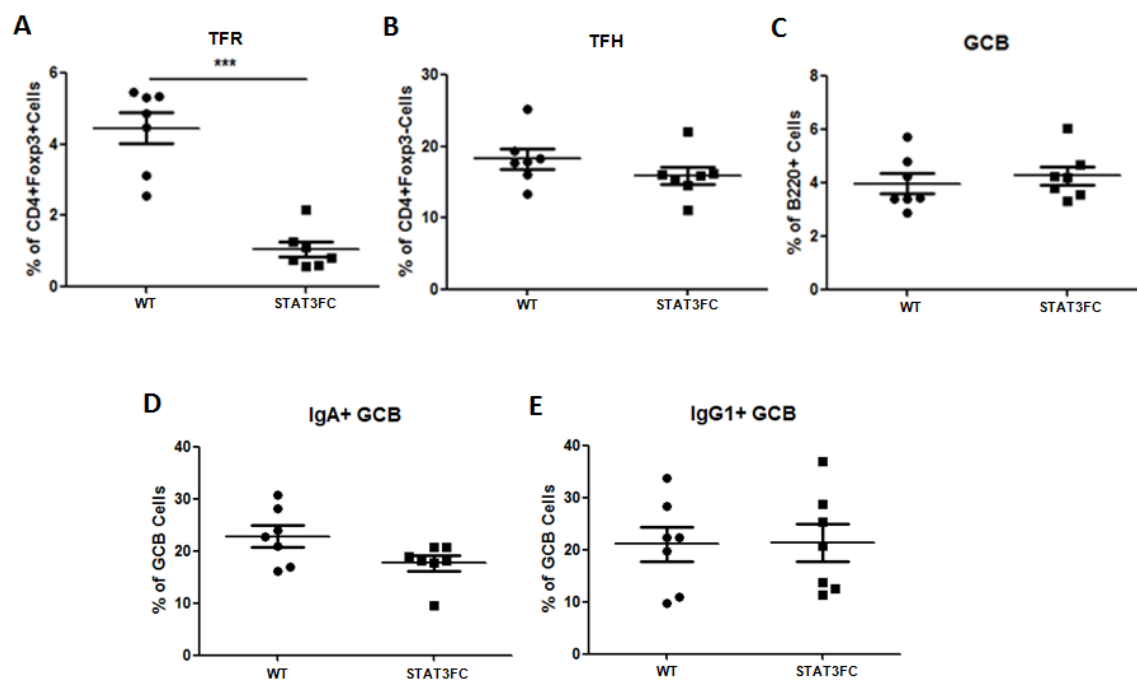


Figure 34. In Peyer's patches, Treg cell specific STAT3 deletion leads to the loss of TFR cells and does not have an effect on TFH, GC B cells, as well as IgG1⁺ and IgA⁺ GC B cells.

PPs from control and STAT3FC mice were isolated and homogenized into single cell suspensions for flow cytometric analysis. The percentages of TFR cells (A), TFH cells (B), GC B cells (C), IgA⁺ GC B cells (D) and IgG1⁺ GC B cells (E) are shown. Graphs show mean +/- SEM, n = 7. *** p < 0.001 (student t test). Data are combined from two independent experiments.

4.4: Discussion.

In this study, we found that STAT3 is required for TFR cell differentiation in spleen after antigen immunization as well as in PPs that receive constitutive antigen stimulation from gut microbiota. Mice with deletion of STAT3 in Treg cells still have normal differentiation of TFH and GC B cells, however the production of antigen-specific IgG1 and IgG2b antibodies is strongly increased in these mice. In addition, switching to IgA and IgG1 in PP GC B cells was not affected by the loss of TFR cells due to Treg-specific STAT3 deletion. Thus, our study has unveiled an essential role for STAT3 in TFR cell differentiation as well as revealed the complex regulatory function of TFR cells on antibody production.

Treg cells and effector T cells in a given immune response experience the same immunological microenvironment including cytokine signals, and therefore, similar to effector T cells, Treg cells express certain lineage determining transcription factors, like Bcl6, Tbet, IRF4 and STAT3 [147], to promote their lineage-specific suppression function. For TFR and TFH cells, they share similar differentiation process, activated by DCs in the T cell zone and maintained by B cells in the GC [148]. They express similar signature genes, CXCR5, PD-1, ICOS and Bcl6, which indicates that similar upstream genes or signaling might be involved in both TFR and TFH cell differentiation. Since the T cell zone and the GC are rich in IL-6 and IL-21, STAT3 signaling is one of the potential lineage determining pathways shared by TFH cells and TFR cells. Here we found that, in both an antigen immunization model and in PPs, STAT3 is indispensable for TFR cell differentiation. Ding et al [140] showed that IL-21 can suppress TFR cell

differentiation while promoting TFH cell differentiation in BXD2 autoimmune mice. However, since STAT1 and STAT3 can be downstream of IL-21 receptor signaling [149], STAT1 may inhibit the differentiation of TFR cells while STAT3 promotes TFR differentiation. Another possibility is that chronic inflammation in BXD2 mice causes the expansion of TFH but not TFR cells, and thus IL-21 does not truly inhibit TFR cell differentiation. Of note, our data from chimeric mice suggest that TFR cells are intrinsically more dependent on STAT3 signaling for differentiation than TFH cells. Our data indicate that in STAT3-deficient conventional CD4⁺ T cells, Bcl6 still can be up-regulated by STAT1 or STAT4 to allow significant TFH cell differentiation. However, in STAT3-deficient Treg cells, STAT1 or STAT4 cannot compensate the TFR cell differentiation defect in the absence of STAT3.

Chaudhry et al reported that Treg cell specific deletion of STAT3 in mice can lead to severe autoimmune diseases in multiple organs by 12-14 weeks of age [89]. However, similar to what was observed with Kluger et al [150, 151], our STAT3^{fl/fl} mice are healthy, fertile, have normal sized lymphoid organs and do not display abnormal inflammation up to 5 months of age. One difference with our work and Chaudhry et al is that we use different strains of STAT3^{fl/fl} mice. Exons 16-21 of STAT3 locus are floxed in our STAT3^{fl/fl} mice [81], whereas exons 21-22 are floxed in Chaudhry et al's STAT3^{fl/fl} mice [152]. However, both types of STAT3 deletions lead to complete loss of STAT3 function [81, 152]. Thus, a likely explanation is that different housing conditions, differences in specific pathogen-

free conditions or differences in endogenous flora in the animal facilities lead to phenotypic differences for similar strains of mutant mice [153].

Our data here with STAT3FC mice helps explain some of the findings of Kluger et al, who used similar mice with conditional deletion of STAT3 by Foxp3-cre to investigate autoimmune kidney disease [150, 151]. For ease of discussion, we will also term these Kluger et al conditional STAT3 knockout mice as “STAT3FC”. In one study with these mice, crescentic glomerulonephritis was induced by injection of nephrotoxic sheep serum [151]. In this study, higher levels of disease were seen in the STAT3FC mice, along with elevated levels of sheep Ig-reactive IgG2b and IgG2c. Our data presented here indicate that these increased IgG responses were likely due to the loss of TFR cells. Interestingly, IgG2b was elevated in STAT3FC mice in both our study and the sheep serum study [151], and IgG2b switching is known to be promoted by TGF-beta signaling [154]. Increased TGF-beta was observed in the STAT3FC mice by Kluger et al [151], which may explain the increased IgG2b responses in these mice. In a second study, pristane was used to induce lupus-like nephritis [150]. In contrast to the crescentic glomerulonephritis study [151], auto-immune Abs induced by pristane were similar or significantly lower in STAT3FC mice compared to controls, despite increased kidney disease, increased Th17 responses and greatly increased death from pulmonary vasculitis in the STAT3FC mice [150]. The question then arises: why weren't increased IgG responses seen in the pristane model? We have data that the repressive function of STAT3-dependent TFR cells on IgG responses can depend on the type of antigen used. We tested

an HIV gp120 prime-boost immunization model in the STAT3FC mice but observed no differences in overall anti-gp120 IgG titer or affinity (data not shown). The HIV gp120 immunization model involves multiple injections over several months [110, 145], and like pristane injection, is more like a chronic immune response than an acute immunization response. Thus, STAT3-dependent TFR cells may play a more critical role in limiting Ig production during acute immune responses than in chronic immune responses

Other studies on TFR cells have observed increased TFH and GC B cell responses in the absence of TFR cells [60-62]. However, our STAT3FC mice have normal sizes of TFH and GC B cell populations both in spleen after immunization and in PPs. Our findings here showing limited TFR cell effects on TFH and GC B cells are consistent with a separate study of TFR cell function we conducted using Bcl6FC mice. Bcl6FC mice also have a severe loss of TFR cells yet have similar sizes of TFH and GC B cell populations compared to their WT controls [145]. One possibility is that the non-physiological conditions caused by T cell transfer into T cell deficient mice, and the non-specific effects of total Treg cell depletion led to the enhanced TFH and GC B cell responses observed in earlier studies of TFR cells.

With STAT3FC mice, we see increased production of SRBC-specific IgG antibodies, without a change in SRBC-specific IgA. This contrasts with Bcl6FC mice, where we observed decreased SRBC-specific IgG and increased SRBC-specific IgA along with decreased TFR cells [145]. The reason for this difference is not yet clear, but likely relates to how STAT3-dependent TFR cells act on TFH

cells compared to Bcl6-dependent TFR cells. With Bcl6FC mice, we observed increased cytokine production from TFH cells, whereas this was not observed in STAT3FC mice (data not shown). The increased cytokines made by TFH cells in Bcl6FC mice likely can explain the altered IgG and IgA responses in these mice. A skewing from IgG to IgA responses may explain the decreased IgG in Bcl6FC mice. Why loss of TFR cells in Bcl6FC mice, but not in STAT3FC mice, leads to alterations in cytokine production by TFH cells requires further in-depth study. Overall, TFH cells appear to be regulated in slightly different ways by STAT3-dependent TFR cells versus Bcl6-dependent TFR cells. However, our data are consistent in showing that TFR cells do not significantly control the proliferation of TFH and GC B cells in the GC reaction, but rather that TFR cells regulate the production of antibodies by GC B cells, and that TFR cells regulate the production of specific Ig isotypes.

Recent reports revealed that TFR cells derived from Treg cells, and TFH cells derived from Treg cells or Th17 cells are important for healthy microbiota in the gut, by supporting the production of diverse IgA Abs [142, 143]. However, we did not see any changes of total IgA⁺ GC B cells in PP from STAT3FC mice. Since TFR cells in the PP can regulate the diversity of gut microbiota via modulation of antigen specific IgA [155], we evaluated the impact of loss of TFR cells in PPs on gut microbiota in STAT3FC mice. We collected cecal contents from WT and STAT3FC mice to analyze the gut bacterial composition by 16s rRNA sequencing. However, we were not able to detect a significant difference in

bacterial diversity in the gut in STAT3^{FC} mice using this approach (data not shown).

In summary, our study demonstrates the important role of STAT3 in TFR cell differentiation. We show that STAT3 in Treg cells is required for the suppression of antigen-specific antibody production, whereas TFR cells do not regulate the sizes of TFH and GC B cell responses. Further research on the role of STAT3 and Bcl6 in Treg cells on the regulation of humoral responses by TFR cells is warranted.

Chapter 5 – Conclusions and future directions

5.1: Conclusions.

5.1.1: STAT3 intrinsically inhibit IL-4 and Bcl6 expression in TFH cells.

In chapter 2, we found that STAT3 can intrinsically inhibit IL-4 and Bcl6 expression in TFH cells. STAT3 function in TFH cells appears to be strongly dependent on the immune environment. In PPs, STAT3 suppressed IL-4 production in TFH cells and in turn suppressed IgG1 class switching of GC B cells, whereas, in spleen following SRBC immunization, the increase of IL-4 expression in STAT3-deficient TFH cells did not lead to the increase of IgG1 class switching of GC B cells. TFH cells have recently emerged as alternative major producers of IL-4 to regulate type 2 humoral immune responses, besides Th2 cells. IL-4 plays an important role in protective immunity against extracellular parasites and is also known to be a key player in allergic disease, such as asthma. TFH and Th2 cells utilize different mechanisms to regulate IL-4 production [97, 98]. The regulation of IL-4 expression in Th2 cells has been extensively characterized, however how IL-4 expression is regulated in TFH cells is not well understood. My dissertation work revealed a novel role of STAT3 in negatively regulating IL-4 production in TFH cells. Mechanistically, the suppression function of Bcl6 on IL-4 requires STAT3 function. STAT3 cooperates with Bcl6 to strongly suppress IL-4, in contrast to repression of IL-10 and IL-13 expression by Bcl6, which is relatively independent of STAT3. Our findings in

STAT3KO mice indicate that TFH cells in HIES patients may have increased IL-4 production as well that leads to high serum IgE level.

STAT3 signaling downstream of IL-6 and IL-21 induces the TFH master transcription factor Bcl6 to promote TFH cell differentiation and function, independent of Th1, Th2 and Th17 cell lineages [55]. The work of Ray et al [56] showed that STAT3 not only activates Bcl6 expression, but also represses the Type I IFN pathway that induces partial Th1 cell differentiation in TFH cells, in the context of acute LCMV infection. However, we found that Bcl6 expression was increased in STAT3-deficient TFH cells in immune settings we tested where type I IFN was not produced. We hypothesize that, in the absence of STAT3, other STATs, such as STAT1 and STAT4, may bind to the Bcl6 promoter and more strongly activate Bcl6 transcription. Therefore, during vaccine design, the activation of certain STATs signaling pathways may be beneficial.

In summary, we have revealed a much more complete picture of how STAT3 regulates TFH cell development and function. Our data suggest a critical role for STAT3 antagonizing the activity of other STAT factors that are potentially activated in CD4 T cells during the immune responses. Notably, STAT3 can act in TFH cells to either repress Bcl6 or to activate Bcl6, possibly depending on the activation of other STAT factors.

5.1.2: TFR cells repress cytokine production by TFH cells and optimize IgG response.

TFH cells provide crucial help to GC B cells for proper antibody production, and a specialized subset of regulatory T cells, TFR cells, modulate this process. However TFR-cell function in the GC is not well understood. In chapter 3, we define TFR cells as a $CD4^{+}$ $Foxp3^{+}$ $CXCR5^{hi}$ $PD-1^{hi}$ $CD25^{low}$ $TIGIT^{high}$ T-cell population. Furthermore, we have used a novel mouse model (“Bcl6FC”) to delete the Bcl6 gene in $Foxp3^{+}$ T cells and thus specifically deplete TFR cells. Following immunization, Bcl6FC mice develop normal TFH- and GC B-cell populations. However, Bcl6FC mice produce altered antigen-specific antibody responses, with reduced titers of IgG and increased IgA. Bcl6FC mice also developed IgG antibodies with significantly decreased avidity to antigen in an HIV-1 gp120 “prime-boost” vaccine model. In an autoimmune lupus model, we observed strongly elevated anti-DNA IgA titers in Bcl6FC mice. Additionally, TFH cells from Bcl6FC mice consistently produce higher levels of IFN- γ , IL-10 and IL-21. Loss of TFR cells therefore leads to highly abnormal TFH-cell and GC B-cell responses (Fig. 36). Overall, our study has uncovered unique regulatory roles for TFR cells in the GC response. And our findings suggest that TFR cells have therapeutic potential for autoimmune diseases, such as SLE, due to their auto-reactive IgA antibody suppression function.

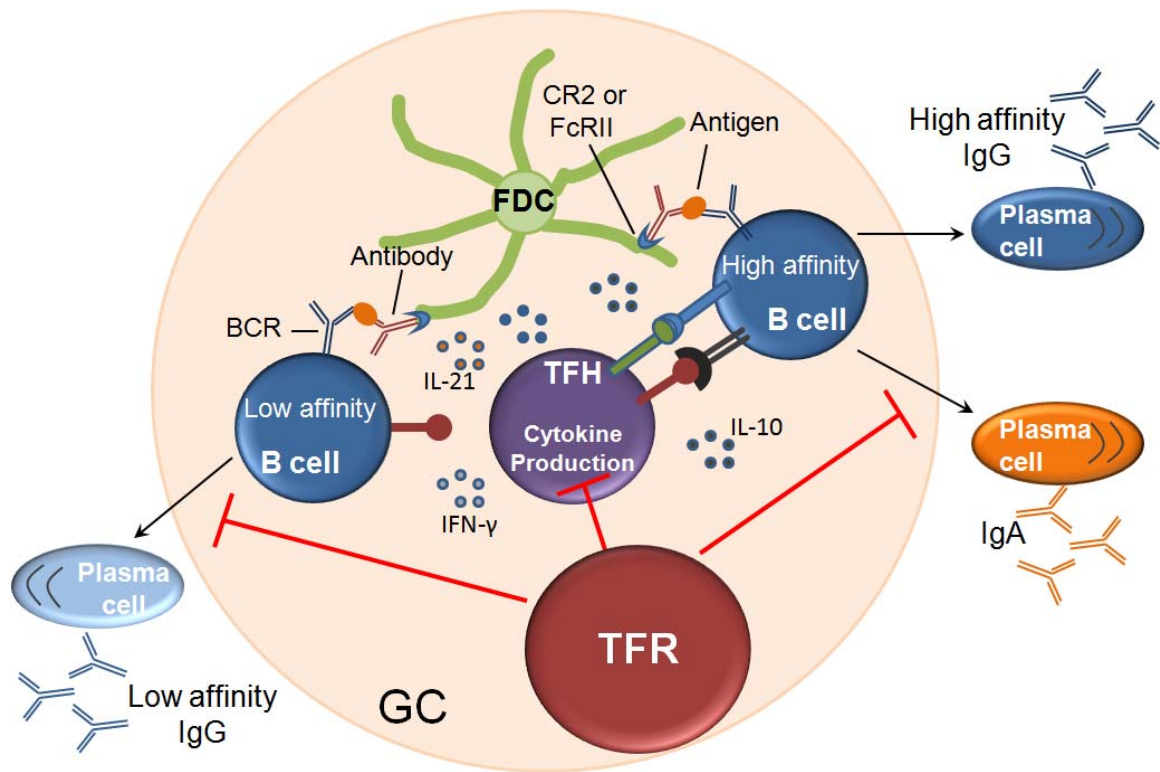


Figure 35. Schematic representation of the regulation of GC reaction by TFR cells.

IL-21 is an important cytokine for B cell survival. TFR cells suppress IL-21 production in TFH cells and in turn inhibit the expansion of low affinity B cell clones. TFR cells also can inhibit the production of antigen specific IgA antibody, possibly by restraining the IL-10 production by TFH cells.

5.1.3: STAT3 is indispensable for TFR cell differentiation.

How TFR cells develop and how their suppressive activity functions are not well understood. In chapter 4, we found that STAT3 is indispensable for TFR cell differentiation. After immunization with SRBC, the loss of TFR cells caused by deletion of STAT3 in Treg cells does not affect the size of TFH or GC B cell population, but rather leads to strongly enhanced production of antigen-specific IgG1 and IgG2b. In Peyer's patches (PPs) in the gut, we found that STAT3 expression in Treg cells is also required for TFR cell formation to commensal organisms. However, loss of TFR cells in the gut did not affect the numbers of TFH cells and GC B cells, nor affect IgG1 or IgA switching by GC B cells. Overall, our study has uncovered unique roles of STAT3 in TFR cell differentiation and the regulation of the antibody response.

5.2: Future directions.

5.2.1: Future directions for chapter 2.

Recently, Sahoo et al [156] found that Batf is important for IL-4 expression in TFH cells. Batf can bind to the CNS2 region of the Il4 locus which is specifically important for Il4 gene transcription in TFH cells [97, 98]. In STAT3-deficient TFH cells, there may be more Gata3 or Batf binding to the CNS2 region of the Il4 locus. Another possibility is that Bcl6 may directly bind to this region and suppress the transcription of Il4 and that STAT3 may be required for the direct binding of Bcl6 to the CNS2 region.

To investigate this aim, we should sort out TFH cells by flow cytometry from control and STAT3KO mice after SRBC immunization and check the binding of Bcl6, Gata3 and Batf at the CNS2 region of the Il4 locus.

5.2.2: Future directions for chapter 3 and 4.

With STAT3FC mice, we see increased production of SRBC-specific IgG antibodies, without a change in SRBC-specific IgA. This contrasts with Bcl6FC mice, where we observed decreased SRBC-specific IgG and increased SRBC-specific IgA along with decreased TFR cells [145]. The reason for this difference is not yet clear, but likely relates to how STAT3-dependent TFR cells act on TFH cells compared to Bcl6-dependent TFR cells. To test this idea, we would like to use a TFH/Treg/B cell co-culture system to study the effect of loss of Bcl6 and STAT3 in Treg cells on the antibody production and class switching. We will sort out TFH cells from WT mice following OVA/Alum immunization, and then culture these TFH cells with B cells with or without control or Bcl6 or STAT3-deficient Treg cells. In this setting, we hope to study how Bcl6 or STAT3-deficient Treg cells affect cytokine production in TFH cells and the antibody production in B cells.

Auto-reactive antibodies are involved in the pathogenesis of several autoimmune diseases, such as SLE and rheumatoid arthritis. These patients have uncontrolled effector T cell responses against self antigen, partially due to the defect in Treg cell function. By using Bcl6FC and STAT3FC mice, we would like to further study the role of TFR cells in the controlling of GC responses in

autoimmune disease models, such as a chromatin immunization model or experimental autoimmune encephalomyelitis. By using these autoimmune diseases models, we also would like to explore the therapeutic potential of TFR cells for autoimmune diseases.

Recent reports revealed that TFR cells derived from Treg cells, and TFH cells derived from Treg cells or Th17 cells are important for healthy microbiota in the gut, by supporting the production of diverse IgA Abs [142, 143]. It is intriguing to study the role of Bcl6 and STAT3 in Treg cells on the regulation of microbiota in the gut. Since we did not see any changes of microbiota composition in the absence of TFR cells at steady state by 16s rRNA sequencing analysis, we could use trinitrobenzene sulfonic acid induced inflammatory bowel disease model with our Bcl6^{FC} mice and STAT3^{FC} mice to study how TFR cells regulate gut microbiota under inflammatory condition.

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Publications

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Honors and Awards

AAI Young Investigator Award, Autumn Immunology Conference	2015
Travel Award, Annual Meeting of the Federation of Clinical Immunology Societies (FOCIS)	2015
AAI Trainee Poster Award, American Association of Immunologists Annual Meeting	2013
Graduate Fellowship, Indiana University School of Medicine BioMedical Gateway (IBMG)	2010